REMARKS

STATUS OF THE CLAIMS:

Claims 20 to 27, and Claims 30 to 36 are pending.

I. Miscellaneous

Objections To Claims

The Examiner has objected to Claims 22, 24, and 26 alleging that they are "dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims". Applicants believe the Examiners objection of Claims 22, 24, and 26 has been overcome in consideration of the arguments presented *infra*.

II. Rejections under 35 U.S.C. § 101

a. The Examiner has rejected Claims 20, 21, 23, 25, 27 and 30 to 36 under 35 U.S.C. § 101, for failure to demonstrate a credible, substantial, specific, or a well-established utility. More particularly, the Examiner alleges that "The specific and substantial utility established by the Rappold-Hoerbrand reference is a diagnosis use based on that the gene is responsible for disorders relating to ataxia as the chromosomal breakpoint of the patient having ataxia is found to reside within the genomic locus of said gene, which is demonstrated by restriction enzyme analysis of the ataxia cDNA, wherein a band shift was observed in the patient, but not in healthy controls (page 5, the third and fourth paragraphs). Therefore, the diagnosis use of the gene depends upon the specific pattern generated by restriction enzyme analysis. Such a specific pattern of a nucleic acid is determined by the unique sequence of the nucleic acid. A limitation of an isolated polynucleotide *encoding* a polypeptide" in the present claims (parts (a)-(c) of claim 20, for example) reads on the disclosed specific polynucleotide of SEQ ID NO:3, which encodes the polypeptide of SEQ ID NO:4, as well as polynucleotides with all possible degeneracy and encoding SEQ ID NO:4. The later would be highly likely to generate different patterns from that of the prior art when subjected to the restriction enzyme digestion, which would not reflect the association to said disorder. As such, those polynucleotides with the sequence degeneracy would not be suitable for the diagnosis use established by the prior art. Further, neither the prior art nor the present specification teaches specifically the use of those polynucleotides for the purpose of diagnosis or any other purpose. Thus, there was no immediately apparent or "real world" utility for those polynucleotides, and the claimed invention is incomplete as of the filing date. One may argue that the polynucleotides with the degenerate sequences can be used for the production of the polypeptide. However, as of the filing

date of the present application, no specific and substantial utility for the polypeptide was established or disclosed by the instant specification. Until a specific and substantial utility can be attributed to the HGRA4sv polypeptide, use of a nucleic acid for the production of the protein is not considered by the Patent Office to be a specific or substantial utility, as such use could be asserted for any cDNA."

Applicants disagree and adamantly assert that the HGRA4sv polypeptide has a specific, substantial and credible utility, and that the utility of this polypeptide is the same as the utility established for the encoding HGRA4sv polynucleotide sequence as described in the specification as originally filed. As Applicants established in the February 19th, 2004 Reply to the Office Action mailed on August 20, 2003, the HGRA4 polynucleotide has a patentable utility based upon the prior teachings of Rappold-Hoerbrand (International Publication No. WO 00/58461), which discloses a gene positively associated with ataxia and that encodes a protein that is 100% identical to the HGRA4 polypeptide. The gene encoding the HGRA4 protein was found to have a patentable utility based upon the determination that the genomic region for the HGRA4 gene, referred to as PAC clone 1055C14 by Rappold-Hoerbrand, covers a breakpoint between Xp22 and Xq22 from a ten year old boy suffering from slight mental retardation and severe cerebellar ataxia. As acknowledged by the Examiner, such knowledge can be used to diagnosis patients having such ataxias by comparing the FISH pattern of chromosome spreads of normal patients relative to ataxia patients at this locus.

The Examiner alleges that "the diagnosis use of the gene depends upon the specific pattern generated by restriction enzyme analysis. Such a specific pattern of a nucleic acid is determined by the unique sequence of the nucleic acid. A limitation of 'an isolated polynucleotide encoding a polypeptide' in the present claims (parts (a)-(c) of claim 20, for example) reads on the disclosed specific polynucleotide of SEQ ID NO:3, which encodes the polypeptide of SEQ ID NO:4, as well as polynucleotides with all possible degeneracy and encoding SEQ ID NO:4. The later would be highly likely to generate different patterns from that of the prior are when subjected to the restriction enzyme digestion, which would not reflect the association to said disorder. As such, those polynucleotides with the sequence degeneracy would not be suitable for the diagnosis use established by the prior art."

Applicants do not refute that changing the nucleotide sequence of a given polynucleotide sequence may alter one or more restriction enzyme sites which could affect the restriction digest banding pattern of such a polynucleotide. However, Applicants believe the Examiner has

misconstrued the fact pattern and believe the conclusions drawn are in error and not applicable to the instant invention. Specifically, Rappold-Hoerbrand do not teach that restriction sites are an essential element in the use of the HGRA4 gene in diagnosing ataxias. Rather, Rappold-Hoerbrand relied upon "FisH-analysis on metaphase spreads" (see third paragraph on page 5 of International Publication No. WO 00/58461) to identify the ataxia association of the HGRA4 gene. Applicants point out that FISH analysis does not require use of restriction endonucleases, nor does it rely upon the creation and identification of restriction bands (See Joos et al., J. Biotech., 35:135-153 (1994); submitted concurrently herewith). Rather, as the name implies, FISH (Florescence In Situ Hybridization) analysis relies upon hybridization of a labeled probe sequence to a target sequence and the subsequent imaging of the same.

In the case of Rappold-Hoerbrand, "FISH studies using appropriate cosmids were carried out according to published methods (Lichter and Cremer, 1992). In short, one microgram of the respective cosmid clone was labeled with biotin and hybridized to human metaphase chromosomes under conditions that suppress signals from repetitive DNA sequences.. Detection of the hybridization signal was via FITC-conjugated avidin. Images of FITC were taken by using a cooled charge coupled device camera system" (see Example 2 on page 10 to 11 of International Publication No. WO 00/58461). The Examiner would appreciate that hybridization is very tolerant to nucleotide changes between any two sequences and is limited only according to the hybridization conditions used and the length of the probe sequence. Applicants point out that Rappold-Hoerbrand performed hybridization "under conditions that suppress signals from repetitive DNA sequences" and refer to Lichter and Cremer, 1992 for these conditions. Applicants note that Lichter and Cremer did not publish a joint manuscript during the cited year (e.g., 1992), but rather did publish several seminole papers on in situ hybridization of chromosomes in 1988 (copies of several of these papers are submitted concurrently herewith for the Examiners convenience).

The conditions published by Lichter and Cremer in 1988 (Proc. Natl. Acad. Sci. USA, 85:9664-9668 (1988); submitted concurrently herewith) were as follows: "...The hybridization cocktail also contained 50% formamide, 0.30M NaCL, 0.03 M sodium citrate (pH 7), 10% (wt/vol) dextran sulfate, and on occasion 0.5 mg of sonicated salmon sperm DNA per ml. Simultaneous denaturation of probe and target DNA was carried out at 75°C for 6 min (metaphase spreads) or 94°C for 11 min (tissue slices). Hybridization reactions were incubated at 37°C overnight...Posthybridization washes, detection of hybridized probe using either alkaline

phosphatase-conjugated avidin or fluorescein isothiocyanonate-conjugated avidin, and photographic conditions were as described (14)."

The referenced article 14 from the Lichter and Cremer 1988 paper (Proc. Natl. Acad. Sci. USA, 85:9664-9668 (1988); submitted concurrently herewith) refers to another Lichter and Cremer manuscript published in Human Genetics (Hum Genet., 80(3):224-34 (1988); submitted concurrently herewith) and describes posthybridization washes as being performed as follows: "After hybridization, the slides were washed in 50% formamide, 2xSSC (3x5 min, 42°C) followed by washes in 0.1xSSC (3x5 min, 60°C)" (see the section entitled, "Detection" at bottom of p.225).

According to guidance taught by Kadkol et al (see Mol. Diag., 4(3):169-183 (1999); submitted concurrently herewith), one skilled in the art would appreciate that polynucleotides encoding the HGRA4sv polypeptide would be expected to hybridize to the genomic HGRA4 sequence using the hybridization conditions utilized by Rappold-Hoerbrand and described by Lichter and Cremer. The latter is supported by the fact that the conditions utilized are best described as "moderate" in stringency on account of the relatively long length of the probe ("Biotinylated cosmid DNA (insert size 32 – 45 kb) or cosmid fragments (10-16 kb)" see Rappold-Hoerbrand, p.11), the relatively low temperature of hybridization (37°C – see *supra*), the moderate level of formamide (50% - see *supra*), and the relatively high concentration of salt utilized for this hybridization (0.30M NaCL – see *supra*; see also Kadkol et al, page 174 – "Increasing salt concentration or decreasing temperature favors hybridization, whereas decreasing salt concentration or increasing temperature destabilizes the duplexes"). The length of the probe sequence used by Rappold-Hoerbrand is also important in appreciating why these conditions are "moderate" in stringency since the conditions utilized by Rappold-Hoerbrand and described by Lichter and Cremer were recommended for DNA probes "smaller than 500 nucleotides" (see section entitled, "Probe size" on page 225 of Lichter et al., Hum Genet., 80(3):224-34 (1988)), and specifically for probes "150-250 nucleotides" (see section entitled, "In Situ Hybridization" on page 9664 and 9665 of Lichter et al., Proc. Natl. Acad. Sci. USA, 85:9664-9668 (1988)), while the probe length actually utilized was at least 10 kb in length (see *supra*). Clearly such a long probe length would ensure hybridization between a polynucleotide encoding the HGRA4sv polypeptide and the genomic sequence of HGRA4 would still occur under these conditions despite subtle differences in matching nucleotides. According to Kadkol et al (see Mol. Diag., 4(3):169-183 (1999)), "The Tm of a double stranded DNA duplex decreases by 1°C to 1.5°C with every 1% decrease in homology for duplexes longer than 100 bp". The length of the Rappold-Hoerbrand would have the effect of minimizing the percent decrease in homology between

the encoding HGRA4sv polynucleotide sequence and the corresponding HGRA4 genomic sequence. As a consequence, Applicants assert one skilled in the art would reasonably expect that a polynucleotide encoding a polypeptide of parts (a)-(c) of claim 20 would hybridize to the genomic locus of the HGRA4 gene and would be useful in diagnosing ataxia under these conditions.

Moreover, Applicants also remind the Examiner that Rappold-Hoerbrand et al teach that the genomic region for the HGRA4 gene covers a breakpoint between Xp22 and Xq22, and that the results of the Rappold-Hoerbrand et al experiment "shows clearly that the breakpoint of the patient resides within the genomic locus of the ataxia gene" (see page 5, last sentence of third paragraph). Since the chromosome breakpoint resides within the HGRA4 genomic sequence, one skilled in the art would clearly appreciate that such a disruption in the HGRA4 genomic sequence would result in loss of the HGRA4 protein and that loss of the HGRA4 protein is causative to the incidence of ataxia. The basis for the latter constitutes a basic, fundamental tenet in molecular biology that teaches that a single gene is responsible for the production of a single protein (e.g., the so called "one gene : one enzyme hypothesis"). Thus, it follows that if a gene has been associated with the incidence of a disease or disorder, then the protein encoded by that gene is also directly implicated in the incidence of the same disease or disorder since the latter is derived from the former.

Single protein loss / disease associations are common amongst the known ataxias (see Klockgether and Evert, TNS, 9:413-418 (1998); submitted concurrently herewith) and typically results in "loss of a protein that is essential for the function and survival of a specific population of neurons", in the case of autosomal recessive ataxias (see page 413), or due "to a novel deleterious function of the extended polyglutamine sequences within the proteins encoded by the respective genes" in the case of dominant ataxias (see page 413). In the case of the latter, the polyglutamine tract within these proteins appears to "catalyze aggregation of fragmented and full-length protein" (see page 416) "with nuclear proteins that are specifically expressed in the brain regions affected..." (see page 416) and results in loss of the functional protein. In consideration of the knowledge available to the skilled artisan regarding ataxia incidence as well as basic concepts in molecular genetics, Applicants assert that one skilled in the art would readily appreciate that the analysis of HGRA4 protein expression would also represent a useful tool for the diagnosis of cerebral ataxias since loss of HGRA4 protein expression would be diagnostic for the severe cerebellar ataxia described by Rappold-Hoerbrand. Clearly, such a utility is not limited solely to the ataxia gene itself.

Applicants further point out to the Examiner that Rappold-Hoerbrand appreciated the link between the loss of HGRA4 protein expression to the incidence of ataxia by stating that the subject "of the present invention are further ataxia proteins which are expressed after transcription of the ataxia gene into RNA or mRNA and which can be used in the therapeutic treatment of disorders related to mutations in said genes. The invention further relates to appropriate cDNA sequences which can be used for the preparation of recombinant proteins suitable for the treatment of such disorders." (see page 6, second paragraph), in addition to stating another subject "of the invention are further plasmid vectors for the expression of the DNA of these genes and appropriate cells containing such DNAs. It is a further subject of the present invention to provide means and methods for the genetic treatment of such disorders in the area of molecular medicine using an expression plasmid prepared by incorporating the nucleic acid molecules of this invention downstream from an expression promotor which effects expression in a mammalian host cell" (see page 6, third paragraph).

As a consequence, it is clear that both the polynucleotide sequence encoding the HGRA4 polypeptide, as well as the HGRA4 polypeptide itself has a specific, credible, and well-established utility in diagnosing neural ataxias with each having a separately measurable, but related end-point. In the case of the polynucleotide, the method of diagnosis encompasses changes in chromosome hybridization patterns, while methods of diagnosis using the polypeptide encompasses measuring the loss of expression of the same. Further, since the "utility for the polypeptide" has been established, Applicants assert that polynucleotides encoding the polypeptides of parts (a)-(c) of claim 20, as well as polynucleotides with all possible degeneracy, have a specific, credible, and well-established utility.

As Applicants pointed out in the February 19th, 2004 Reply to the Office Action mailed on August 20, 2003, since the HGRA4 polynucleotide and polypeptide have a specific, substantial, and credible or well-established utility based upon the teachings of Rappold-Hoerbrand and the arguments presented herein and in prior correspondence before the Office, the HGRA4sv polynucleotide and polypeptide also has a specific, substantial, and credible or well-established utility since the splice variant and its encoding polynucleotides each derive from the same ataxia associated gene (e.g., HGRA4). Moreover, since disruption of the genomic sequence of the HGRA4 gene is expected to result in loss of the HGRA4 polypeptide (see *supra*), one skilled in the art would also expect this gene disruption would result in loss of the HGRA4sv protein as well since the expression of the latter is also dependent upon an intact HGRA4 gene. Applicants believe the

Examiners rejection of Claims 20, 21, 23, 25, 27, and 30 to 36 have been overcome in consideration of the arguments presented herein and request that the Examiner withdrawal the remaining rejections of the same.

Applicants believe that all of the Examiners rejections and objections have been overcome and that all of the pending claims before the Examiner are in condition for allowance. An early Office Action to that effect is, therefore, earnestly solicited.

Applicants reply is timely filed and no extensions of time are required.

If any fee is due in connection herewith not already accounted for, please charge such fee to Deposit Account No. 19-3880 of the undersigned. Furthermore, if any extension of time not already accounted for is required, such extension is hereby petitioned for, and it is requested that any fee due for said extension be charged to the above-stated Deposit Account.

Respectfully submitted,

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64 Llinás, R. and Welsh, J.P. (1993) *Curr. Opin. Neurobiol.* 3, 958–965
65 Zagrebelsky, M. *et al.* (1996) *Eur. J. Neurosci.* 8, 1051–1054
66 Ramakers, G.M. *et al.* (1995) *J. Biol. Chem.* 270, 13892–13898
67 Luthi, A. *et al.* (1994) *Nature* 372, 777–779
68 Schachner, M. (1997) *Curr. Opin. Neurobiol.* 9, 627–634
69 McNamara, R.K. and Lenox, R.H. (1997) *J. Comp. Neurol.* 379, 48–71
70 Sotelo, C. *et al.* (1975) *Brain Res.* 98, 574–581
71 Kleim, J.A. *et al.* (1997) *J. Neurosci.* 17, 717–721
72 Bravin, M. *et al.* (1996) *Eur. J. Neurosci.* 9 (Suppl.), 20.15 (abstract)

Genes involved in hereditary ataxias

Thomas Klockgether and Bernd Evert

The hereditary ataxias are a group of inherited neurodegenerative disorders characterized by progressive ataxia that results from degeneration of the cerebellum and its afferent and efferent connections. Recent molecular research has led not only to the discovery of a number of causative mutations, but also shed light on the likely mechanisms by which these mutations cause the respective phenotypes. In Friedreich's ataxia (FRDA), the most common type of autosomal recessive ataxia, the loss of a mitochondrial protein, frataxin, results in overload of mitochondrial iron and oxidative stress. The autosomal dominant ataxias, spinocerebellar ataxia type 1 (SCA1), SCA2, SCA3 and SCA7, are caused by inheritance of an unstable, expanded CAG trinucleotide repeat. These disorders are assumed to be due to a novel deleterious function of the extended polyglutamine sequences within the proteins encoded by the respective genes. Recent observations in transgenic mice and in human post-mortem tissue suggest that the extended proteins are transported into the nucleus of neurons where they form intranuclear inclusions that disrupt normal nuclear function. In another group of dominant disorders, episodic ataxia type 1 and type 2 (EA-1, EA-2) and SCA6, the mutations affect genes that code for ion channels.

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THE HEREDITARY ATAXIAS are a heterogeneous group of inherited neurodegenerative disorders characterized by progressive ataxia that results from degeneration of the cerebellum and its afferent and efferent connections[1]. Although hereditary ataxias have been known to clinicians for more than a century, investigators in the field of ataxia remained largely ignorant of the underlying pathogenic mechanisms until recently. The situation changed dramatically when the first gene mutations causing hereditary ataxia were identified. At present, more than ten genes involved in human hereditary ataxia have been discovered, and there has also been unexpectedly fast progress towards deciphering the molecular and cellular mechanisms by which the mutations cause neuronal degeneration (Table 1).

Hereditary ataxias are divided into autosomal dominant and autosomal recessive ataxias[18]. The recessive ataxias are caused by the loss of a protein that is essential for the function and survival of a specific population of neurons. As a prototype of this group, Friedreich's ataxia (FRDA), which is the most common type of recessive ataxia, will be discussed in this article. Genetic heterogeneity of dominant ataxias has been established, with disease loci assigned to chromosome 6p (spinocerebellar ataxia type 1, SCA1), 12q (SCA2), 14q (SCA3), 16q (SCA4), 11cen (SCA5), 19p (SCA6) and 3p (SCA7)[7–15]. Some families with dominant transmission of the disease suffer from an episodic type of ataxia. To date, two genetic variants, episodic ataxia type 1 and type 2 (EA-1, EA-2), are known[16,17]. For the dominant disorders, two different pathogenic mechanisms have been identified so far. Disorders of one group, including SCA1, SCA2, SCA3 and SCA7, are caused by inheritance of an unstable, expanded CAG trinucleotide repeat. All available evidence suggests that these disorders are due to a novel deleterious function of the extended polyglutamine sequences within the proteins encoded by the respective genes. In another group of dominant disorders, including EA-1, EA-2 and SCA6, the mutations affect genes that code for ion channels. These disorders add to the growing list of channelopathies among neurological disorders. The causative mutations of SCA4 and SCA5 have not yet been identified.

Friedreich's ataxia

Friedreich's ataxia usually starts around puberty, presenting as progressive ataxia accompanied by areflexia, posterior column signs, and dysarthria. In addition, non-neurological symptoms such as hypertrophic cardiomyopathy and diabetes mellitus can be associated with FRDA[19]. The first pathological changes affect the large sensory neurons of the dorsal root ganglia followed by degeneration of the spinocerebellar, posterior column and pyramidal tracts. Mild degeneration is also observed in the cerebellum.

Ninety-four percent of FRDA patients are homozygous for an expanded intronic GAA repeat in a novel gene, *X25*, while the remaining 6% are compound heterozygotes with an expanded repeat on one allele and a point mutation on the other allele[2]. The number of repeats ranges from 6 to 29 in normals and 120 to 1700

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TABLE 1. Molecular genetics of hereditary ataxias

Disease	Chromosomal localization	Mutation	Gene product	References
Autosomal recessive				
Friedreich's ataxia (FRDA)	9q	GAA repeat	Frataxin	2
Ataxia telangiectasia (AT)	11q	Deletion, insertion	Phosphatidylinositol-3′ kinase	3
Abetalipoproteinemia	4q	Missense mutation	Microsomal triglyceride transfer protein	4
Ataxia with isolated vitamin-E deficiency	8q	Deletion	α-Tocopherol transfer protein	5
Cerebrotendinous xanthomatous	2q	Missense mutation	Sterol 27-hydroxylase	6
Autosomal dominant				
SCA1	6p	CAG repeat	Ataxin-1	7
SCA2	12q	CAG repeat	Ataxin-2	8–10
SCA3	14q	CAG repeat	Ataxin-3	11
SCA4	16q	?	?	12
SCA5	11cen	?	?	13
SCA6	19p	CAG repeat	CACNA1A (Ca^{2+} channel)	14
SCA7	3p	CAG repeat	Ataxin-7	15
EA-1	12p	Missense mutation	KCNA1 (K^+ channel)	16
EA-2	19p	Deletion, splice mutation	CACNA1A (Ca^{2+} channel)	17

in patients[2,19–22]. *X25* encodes a protein of 210 amino acids, frataxin, with an expression pattern that correlates in part with the sites of pathology of the disease[2]. Analysis of frataxin mRNA and protein in humans indicates that expression of the frataxin gene is markedly reduced in FRDA (Refs 2,23). The GAA expansion might affect mRNA stability, splicing efficiency or transcriptional or translational processes, or both, leading to an insufficient production of the protein. Some residual expression remains, depending on the length of the shorter allele. Larger alleles are associated with lower residual levels of frataxin, earlier onset of disease and a more severe phenotype[19,20,23].

A first hint as to the cellular localization of frataxin came from phylogenetic studies showing that the yeast homolog of frataxin contains a mitochondrial-targeting sequence[24,25]. Subsequent studies in human cells using epitope tagging and colocalization with mitochondrial markers confirmed the mitochondrial localization of frataxin[23,24,26,27]. Disruption of the frataxin homolog in yeast results in clones that fail to grow on nonfermentable sources of carbon, such as glycerol, and exhibit a phenotype typical of rho^- mutants defective in mitochondrial DNA (Refs 24,28). Indeed, mutant strains are respiration deficient owing to loss of mitochondrial DNA (Ref. 28). In addition, they are more sensitive to oxidative stress induced by administration of hydrogen peroxide and iron[29]. The mitochondrial defect in frataxin-deficient yeast strains is most probably due to overload of mitochondrial iron. In mutant strains mitochondrial iron content is 10 to 15 times higher than in wild-type strains[27,29]. Iron is an essential component of iron–sulfur clusters in the active sites of enzymes of the mitochondrial respiratory chain. On the other hand, increased iron concentrations might damage mitochondrial DNA and impair mitochondrial function by catalyzing the formation of highly toxic hydroxyl radicals ($OH^·$) from hydrogen peroxide that is produced as a normal byproduct of various enzymatic reactions in the brain. This reaction is known as the Fenton reaction.

If one assumes that the function of human frataxin resembles that of its yeast homolog, FRDA can be characterized as a mitochondrial disease caused by a mutation of the nuclear genome. The hypothesis that FRDA is due to mitochondrial dysfunction and oxidative stress is supported by a number of early biochemical findings showing mildly reduced levels of various respiratory chain enzymes in white blood cells, fibroblasts and post-mortem tissues of FRDA patients[30]. Whereas these studies were performed on nonaffected tissues, a recent study measured the activity of mitochondrial enzymes in heart biopsies. This study revealed a selective reduction of the activity of complex I, II and III of the mitochondrial respiratory chain[31]. The common link between these enzymes is the presence of iron–sulfur clusters. A potential role of oxidative stress in the pathogenesis of FRDA is further suggested by the striking similarity of FRDA to inherited or acquired diseases due to deficiency of the antioxidant defense molecule, vitamin E (Ref. 32).

Despite the enormous progress that has been made in deciphering the molecular cause of FRDA, many questions remain unresolved. Although the distribution of sites of frataxin expression partly matches the sites of pathology there are some major discrepancies. Frataxin is prominently expressed in a number of nonneuronal tissues, such as liver, muscle, thymus and brown fat, that are not affected in FRDA. While the resistance of some of these tissues to the loss of frataxin might lie in their dividing nature it is unclear why skeletal muscle and cerebellar granule cells, which also significantly express frataxin, do not undergo degeneration in FRDA. On the other hand, neuronal populations, such as the corticospinal and spinocerebellar neurons, that are affected in FRDA express frataxin only at a very low level[24]. To account for these discrepancies one has to postulate that the sensitivity of different tissues to frataxin deficiency varies considerably. Although the studies on yeast mutants demonstrate the importance of frataxin for intracellular iron homeostasis its exact physiological role remains to be

established. Another unresolved question concerns the precise mechanism by which the expansion of the intronic GAA repeats causes reduced mRNA levels.

A number of research groups are currently attempting to create mice with a targeted disruption of the *X25* gene. These animals will be of inestimable value in the study of the pathogenesis of FRDA and will help in resolution of the questions outlined above. In addition, studies on these animals will help to answer the question whether neuronal cell death due to frataxin deficiency is an early event of the disease process or whether cell death occurs late and simply serves to eliminate neurons that have lost their normal function. These animal models will also serve to develop and test rational therapeutic strategies for FRDA. Gene therapeutic approaches that aim at replacing the defective gene in affected tissues are envisaged but will have to deal with a number of technical difficulties concerning choice of an appropriate vector system, effective delivery of the transgene and stability of expression. It will be comparatively easy to test whether pharmacological interventions, such as iron chelation, antioxidative treatment, replacement of mitochondrial enzymes or application of antiapoptotic agents, are effective.

CAG repeat disorders

The common clinical features of SCA mutations are progressive ataxia, onset in midlife with large variation and autosomal dominant inheritance. Neuropathologically, they are characterized by loss of cerebellar Purkinje cells. However, the pathological changes often go beyond the cerebellum and can also affect the spinal cord, brainstem, basal ganglia, retina and peripheral nervous system.

Molecular genetic studies have identified five disorders, SCA1, SCA2, SCA3, SCA6 and SCA7, that are caused by inheritance of expansion of CAG trinucleotide repeats, coding for an extended polyglutamine sequence[7–11,14,15]. Larger repeats are associated with earlier onset of disease, more rapid progression, a more severe phenotype, and more extended neuropathology[7,33–35].

In SCA1, SCA2, SCA3 and SCA7, the mutation resembles the previously characterized mutation in Huntington's disease (HD) in that it affects a gene encoding a protein of unknown function with ubiquitous expression. In these disorders, the repeat size ranges from 6 to 34 in normal and 35 to 135 in expanded alleles (Fig. 1). The expanded repeats are subject to changes in length during intergenerational transmission, resulting in anticipation, that is, earlier onset of disease in subsequent generations[36]. Anticipation is a highly characteristic feature of CAG repeat disorders. Observation of this phenomenon in families with an inherited neuropsychiatric disease will therefore arouse the suspicion that expansion of a CAG repeat is the underlying mutation.

The SCA6 mutation is different in a number of aspects. First, the mutation affects a gene with known function, the human α_{1A} voltage-dependent Ca^{2+} channel subunit, CACNA1A, which is expressed in a region-specific way with strongest expression in cerebellar Purkinje cells[38]. Second, the repeats in the *CACNA1A* gene are much shorter, with normal alleles ranging from 4 to 17 and expanded ones from 21 to 30 (Refs 14,39–44). Expanded *SCA6* alleles thus fall within the same size range as normal alleles of the other *SCA* mutations. Third, intergenerational instability has been observed in SCA6 only in rare instances[44].

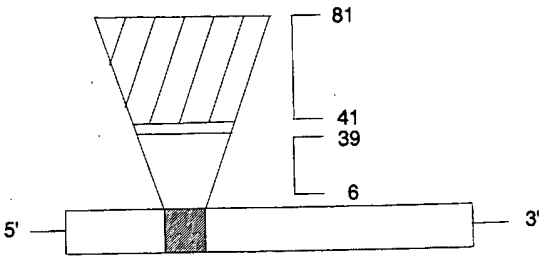

Fig. 1. *Expansion of the CAG repeat in* SCA1. *The coding region of the* SCA1 *gene is indicated by a box; the 5' and 3' untranslated regions are shown as lines. The CAG repeat expansion is represented by the hatched area within the box. While the repeat length in normals varies between 6 and 39 trinucleotides, SCA1 patients have one allele with a range of 41 to 81 repeat units. The mutated* SCA1 *genes contain uninterrupted CAG stretches. In contrast, normal alleles have a midstream CAT interruption[36]. The length of the repeat and the presence or absence of the interruption appear to be crucial for the stability of the trinucleotide repeats. Repeats in the normal size range containing a CAT interruption are stable in parent-to-offspring transmission. Expanded uninterrupted repeats occurring in SCA1 patients are unstable with a tendency to further expansion during meiosis, in particular during spermatogenesis. This mechanism leads to larger expansion in offspring of affected males. There is also mitotic instability of the expanded repeats, leading to varying repeat lengths in different body tissues. A possible explanation for these findings is that frequent cell divisions lead to greater heterogeneity and larger expansions. This applies for spermatozoa, which undergo a greater number of cell divisions than oogonia[37].*

Most researchers assume that the pathogenic mechanism causing neurodegeneration as a result of the *SCA* mutations involves a novel deleterious function of the proteins harboring the extended polyglutamine tract (gain of function) rather than a loss of function of the respective proteins. At present, the evidence for this assumption is only indirect because it is derived from observations in two other CAG repeat disorders, HD and spinobulbar muscular atrophy (SBMA). Individuals with a deletion of the *HD* or androgen-receptor gene – the latter gene carries a CAG repeat expansion in SBMA patients – do not develop HD- or SBMA-like symptoms[45,46]. Similarly, targeted disruption of the *HD* gene in mice does not result in neurodegeneration[47].

Recent research in transgenic mouse models and post-mortem human brains suggests that formation of intranuclear inclusions in affected tissues might be the common pathogenic mechanism in CAG repeat disorders. Studies in transgenic mice that overexpress a mutant human *SCA1* gene showed that ataxin-1, the protein encoded by the *SCA1* gene, localized to a single nuclear stucture of 2 μm in size, while normal ataxin-1 was found throughout the nucleus in smaller structures of 0.5 μm in size. The nuclear inclusions appear weeks before the onset of ataxia and Purkinje cell loss. In brain tissue from SCA1 patients, similar nuclear inclusions were detected in brain regions affected by the disease, but not in other parts of the brain. In nonneuronal cells transfected with the *SCA1* gene, ataxin-1 is associated with the nuclear matrix and causes a specific redistribution of promyelocytic leukemia protein (PML), another nuclear matrix-associated protein[48]. Nuclear inclusions were also demonstrated in brain tissue from HD and SCA3 patients and in transgenic mice expressing a fragment of the HD gene containing an expanded CAG repeat[49,50].

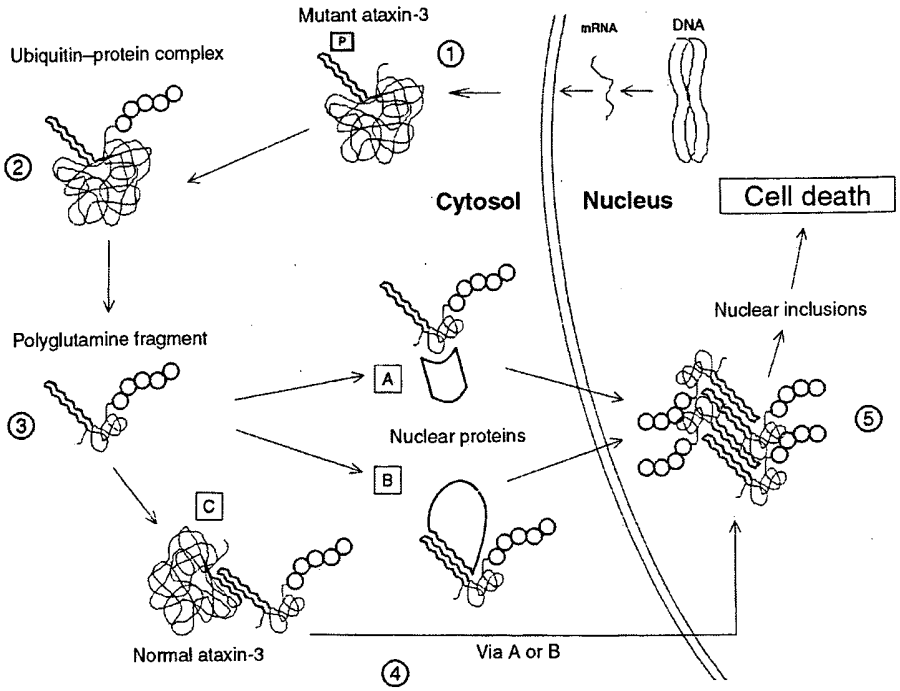

Fig. 2. ***Hypothetical pathogenic mechanism of SCA3.*** *Expression of ataxin-3 with an expanded polyglutamine tract confers a conformational change or misfolding of the nascent polypeptide induced by formation of a stable hairpin (P) with β-sheet structure (red zigzag line) that presents a target for immediate degradation* **(1)**. *The abnormal protein is recognized by the proteolytic machinery and degradation is initiated by the ubiquitin-dependent pathway covalently attaching ubiquitin molecules (blue circles) to the N-terminus, consistent with the finding that expanded ataxin-3 is found in ubiquitinated aggregates* **(2)**. *The mutant protein marked with the branched chain of ubiquitins is cleaved by a protease that cuts the target protein into a series of small fragments. The formation of a polyglutamine-containing proteolytic fragment of the disease protein enables abnormal protein–protein interactions that, in turn, cause the translocation to the nucleus* **(3)**. *The polyglutamine fragment might interact with unknown nuclear proteins containing specific binding motifs for a small portion of degraded ataxin-3* **(A)** *or form polar zippers with other complementary polyglutamine-containing domains of nuclear proteins* **(B)**. *Dimerization also favors a catalytic mechanism so that normal ataxin-3* **(C)**, *which by itself does not form nuclear aggregates, is recruited into insoluble complexes through a direct interaction between the polyglutamine domains* **(4)**. *The formation of insoluble filamentous aggregates might be promoted by transglutaminase-mediated crosslinking generating the nuclear inclusions that disrupt the structural integrity of the nucleus and consequently cause cell death* **(5)**.

At present, it remains unclear which components of the *SCA* gene products are present within the nuclear inclusions. Forced expression of expanded full-length ataxin-3, the protein encoded by the *SCA3* gene, in nonneuronal cells does not lead to a redistribution of the protein, while expression of truncated ataxin-3 containing an extended polyglutamine tract with short flanking sequences localizes to intranuclear and perinuclear bodies similar to the inclusions seen in autopsy material. Truncated ataxin-3 with an expanded repeat, but not full-length ataxin-3, forms insoluble high-molecular weight complexes. However, when truncated and full-length ataxin-3 are coexpressed, full-length ataxin-3 is recruited into the insoluble complexes, suggesting that the polyglutamine fragment serves to catalyze aggregation of fragmented and full-length protein (Fig. 2)[49]. The possibility that the formation of polyglutamine-containing protein fragments is of crucial importance for the pathogenesis of CAG repeat disorders is also suggested by the observation that expression of polyglutamine-containing fragments of ataxin-3 but not of full-length expanded ataxin-3 induces apoptotic cell death[51].

The region-specific neurodegeneration resulting from *SCA* mutations is likely to be determined by interactions of the mutated *SCA* gene products with nuclear proteins that are specifically expressed in the brain regions affected by the respective diseases. In a yeast two-hybrid system, leucine-rich acidic nuclear protein (LANP), a protein that is expressed predominantly in cerebellar Purkinje cells, was shown to interact with ataxin-1. LANP contains five leucine-rich repeats, an amphipathic motif known to mediate protein–protein interactions. The strongest interaction occurred with ataxin-1 containing an extended polyglutamine tract. The interaction of ataxin-1 with LANP might not only explain the region-specific neurodegeneration in SCA1, but might also offer clues to the intracellular mechanisms leading to cell dysfunction and finally death. In nonneuronal cells overexpressing ataxin-1, LANP is recruited to the intranuclear inclusions. Sequestration to the inclusions might interfere with the physiological function of LANP, which still remains undefined[52].

Investigation of the pathogenic effects of nuclear inclusions is one of the most urgent tasks of future studies in CAG repeat disorders. In particular, careful immunocytochemical studies in human post-mortem tissue are required to clarify the precise composition of the inclusions in the various CAG repeat disorders. If these studies confirm that the inclusions contain truncated ataxins, it will be important to investigate the mechanisms by which the ataxins are proteolytically cleaved. Targeted disruption of genes encoding proteins that potentially interact with ataxins will be helpful in elucidating the function of these proteins and in studying the transport of ataxins to the cell nucleus. Although it is generally assumed that the CAG repeat disorders are due to a gain of function, one cannot exclude the possibility that partial loss of their physiological function contributes to the pathogenesis of these disorders. It is conceivable that ataxins might exert a protective action on cerebellar neurons. Future research should therefore attempt to define the physiological role of normal *SCA* genes (Box 1).

Channelopathies

To date, two different ion channel genes are known to be associated with dominantly inherited human cerebellar disease. Missense mutations in a brain K^+ channel gene, *KCNA1*, result in EA-1 (Ref. 16). EA-1 is characterized by brief attacks of ataxia and dysarthria with onset in early childhood. The attacks, which are often provoked by movements and startle, last for seconds to minutes and can occur several times per day. The affected children do not develop persistent ataxia or cerebellar atrophy[53]. Expression studies in *Xenopus*

Box 1. *SCA* genes and normal function of the cerebellum

Although the *SCA* mutations cause degeneration of the cerebellum, all of the available evidence suggests that the physiological function of *SCA* genes is not related directly to the cerebellum. With the exception of the *CACNA1A* gene, which carries the *SCA6* mutation, the *SCA* gene products are expressed ubiquitously with no preference for the CNS or the cerebellum. Unfortunately, our present knowledge of the normal regulation of gene expression and the physiological function of the *SCA* genes is limited. Understandably, the efforts of many researchers are directed towards the elucidation of the pathogenetic mechanisms of the mutations affecting the *SCA* genes, rather than clarifying their nomal function.

There is a transient burst of *SCA1* expression at P14 in the murine cerebellum. This coincides with the period when the murine cerebellar cortex bocomes active functionally. This finding suggests a specific role of *SCA1* in cerebellar development. However, the observed regulation of *SCA1* gene expression is not specific for the cerebellum since there is a similar increase of gene expression in the intervertebral discs of the spinal column. In addition, mice lacking ataxin-1 do not show cerebellar deficits. Instead, these mice have learning deficiencies and decreased hippocampal paired-pulse facilitation, suggesting a role of *SCA1* in hippocampal functions.

Purkinje cells strongly express the pore-forming α_{1A} voltage-dependent calcium-channel subunit encoded by the *CACNA1A* gene. Mutatins of this gene might cause progressive (SCA6) or episodic ataxia (EA-2). It is not quite clear whether the α_{1A} subunit mediates P- or Q-type currents. Therefore, the terminology P/Q-type currents is often used. In Purkinje cells the majority of voltage-gated calcium flux is mediated by P-type channels that play an essential part in the specific electrophysiological characteristics of Purkinje cells, in particular their ability to generate complex spikes. Systematic administration of specific blockers of P-type calcium channel antagonists has been shown to reduce the spontaneous activity of cerebellar Purkinje cells. Altered channel characteristics resulting from mutations of the *CACNA1A* gene are therefore expected to disturb profoundly the normal function of cerebellar Purkinje cells. In addition, a long-lasting increase of calcium influx into Purkinje cells might lead to persistent changes of intracellular signalling and, finally, to cell death.

oocytes suggest that the mutation impairs the capacity of affected neurons to repolarize effectively following an action potential[54].

The other ion channel gene involved in cerebellar disease is *CACNA1A*, encoding the α_{1A} voltage-dependent Ca^{2+} channel subunit. A range of phenotypes is associated with mutations of *CACNA1A*. Four different missense mutations affecting conserved functional domains within the membrane-spanning and pore-forming regions have been found in familial hemiplegic migraine (FHM) (Ref. 17). Some FHM families develop cerebellar atrophy and suffer from mild interictal ataxia. Another missense mutation affecting the pore-forming region resulting in distortion of the central pore was described in a family with rapidly progressive severe ataxia[55]. Mutations causing truncation of the α_{1A} Ca^{2+} channel subunit have been found in families with EA-2 (Ref. 17). Compared with EA-1, attacks in EA-2 start later, last longer and are precipitated by emotional stress and exercise but not by startle. Some individuals, who might or might not suffer from episodic ataxia, have slowly progressive ataxia and cerebellar atrophy[56]. Finally, moderate expansion of a CAG repeat in the aberrantly translated 3′ noncoding region of the *CACNA1A* gene causes SCA6 (Fig. 3)[14]. In contrast to most other *SCA* mutations, the clinical and neuropathological manifestations in SCA6 almost

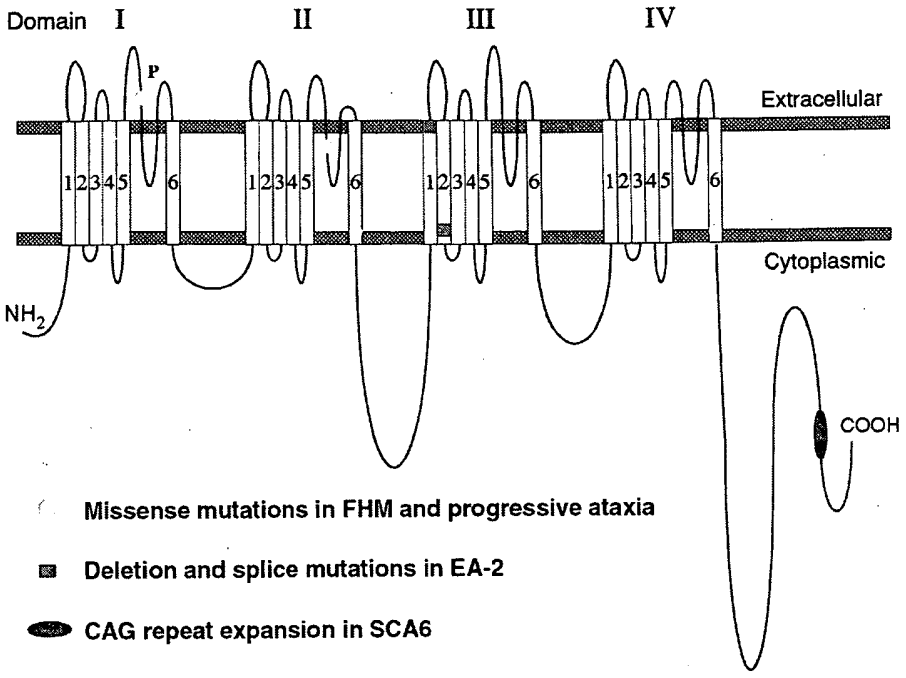

Fig. 3. ***Schematic representation of the* CACNA1A *gene.*** *The* CACNA1A *gene encodes the the α_{1A} voltage-dependent Ca^{2+} channel subunit. Brain voltage-sensitive Ca^{2+} channels are multimeric complexes composed of one member of the α_1-subunit family that forms the ion channel and additional subunits, α_2/δ and β, which have regulatory roles. The different genes encoding the α_1-subunit family are designated A, B, C, D, E and S. The Ca^{2+} channels containing the α_{1A} subunit are thought to mediate P- and Q-type currents. The α_{1A} subunit is expressed throughout the brain with highest expression in cerebellar Purkinje cells. The α_{1A} subunit has four repeated domains, I–IV, each of which contains six membrane-spanning segments, 1–6. The pore, P, is formed by the segments of domains I–IV, which lie between the membrane-spanning segments 5 and 6. There are various splice variants of the α_{1A} subunit. In those splice variants that extend the coding region by 239 amino acids, the CAG repeat in the 3′ end of the gene is translated. Missense mutations (yellow dots) are associated with familial hemiplegic migraine (FHM) and progressive ataxia. Deletions and splice mutations (red squares) are assumed to produce nonfunctional subunits. These mutations cause episodic ataxia type 2 (EA-2) and progressive ataxia. The spinocerebellar ataxia type 6 mutation (SCA6, blue ellipse) is an expanded CAG repeat in the 3′ end of the gene that, if translated, encodes an extended polyglutamine in the intracellular C-terminal portion of the protein. The SCA6 mutation causes an almost pure cerebellar form of progressive ataxia. Some families also have episodic features.*

Outstanding questions

- How does the intronic GAA repeat expansion in the *X25* gene cause reduced expression of frataxin?
- What is the physiological role of frataxin in human tissues?
- What are the consequences of frataxin deficiency for mitochondrial respiration?
- What are the physiological functions of the various ataxins?
- Do the ataxins interact with other cellular proteins and are these interactions essential for the pathogenesis of the disorders?
- Do the intranuclear inclusions found in the brains of SCA patients represent an essential pathogenetic mechanism or are they an epiphenomenon of another unknown process?
- What are the mechanisms of protein aggregation of mutated ataxins and how might aggregation be prevented?
- How do the different mutations of the *CACNA1A* gene affect the physiological function of neuronal calcium channels?
- Does the pathogenesis of SCA6 resemble that of other CAG-repeat disorders or is it related to an altered function of normal calcium channels?

selectively affect the cerebellum[42]. Some SCA6 patients also experience episodic ataxia[57,58].

The episodic features of the disorders due to mutations of the *CACNA1A* gene are most likely due to haploinsufficiency. Formation of truncated proteins in EA-2 is supposed to lead to a reduced number of functional Ca^{2+} channels, resulting in intermittent dysfunction of central neurons. Purkinje cells appear to depend very heavily on normal function of Ca^{2+} channels formed by the α_{1A} voltage-dependent Ca^{2+} channel subunit, explaining why the respective mutations often cause intermittent ataxia[38]. The expanded polyglutamine stretch in *CACNA1A* isoforms in SCA6 might induce an altered protein structure and result in formation of a reduced number of functional channels und thus lead to episodic ataxia. The observation that all known types of mutations affecting the *CACNA1A* gene can lead to cerebellar atrophy and persistent ataxia suggests that cerebellar degeneration is due to dysfunction of Ca^{2+} channels probably resulting in excess entry of Ca^{2+} into neurons. Alternatively, neurodegeneration in SCA6 might be caused by mechanisms similar to those in other CAG repeat disorders. An argument in favor of this hypothesis is that SCA6 shares the inverse relationship of repeat size and age of onset with other CAG repeat disorders[14]. The fact that the extended polyglutamine tract in SCA6 patients is in the same size range as normal polyglutamine tracts encoded by other *SCA* genes does not exclude a gain-of-function mechanism since the toxicity of the polyglutamine might not only depend on the repeat length but also on the protein context. Future studies in transgenic mice expressing mutant alleles of the *CACNA1A* gene will enable the resolution of the mechanism of pathogenesis of SCA6, FHM and EA-2.

Selected references

1 Greenfield, J.G. (1954) *The Spino-Cerebellar Degenerations*, Charles C. Thomas
2 Campuzano, V. *et al.* (1996) *Science* 271, 1423–1427
3 Savitsky, K. *et al.* (1995) *Science* 268, 1749–1753
4 Sharp, D. *et al.* (1993) *Nature* 365, 65–69
5 Ouahchi, K. *et al.* (1995) *Nat. Genet.* 9, 141–145
6 Leitersdorf, E. *et al.* (1993) *J. Clin. Invest.* 91, 2488–2496
7 Orr, H.T. *et al.* (1993) *Nat. Genet.* 4, 221–226
8 Pulst, S.M. *et al.* (1996) *Nat. Genet.* 14, 269–276
9 Sanpei, K. *et al.* (1996) *Nat. Genet.* 14, 277–284
10 Imbert, G. *et al.* (1996) *Nat. Genet.* 14, 285–291
11 Kawaguchi, Y *et al.* (1994) *Nat. Genet.* 8, 221–228
12 Flanigan, K. *et al.* (1996) *Am. J. Hum. Genet.* 59, 392–399
13 Ranum, L.P. *et al.* (1994) *Nat. Genet.* 8, 280–284
14 Zhuchenko, O. *et al.* (1997) *Nat. Genet.* 15, 62–69
15 David, G. *et al.* (1997) *Nat. Genet.* 17, 65–70
16 Browne, D.L. *et al.* (1994) *Nat. Genet.* 8, 136–140
17 Ophoff, R.A. *et al.* (1996) *Cell* 87, 543–552
18 Harding, A.E. (1983) *Lancet* i, 1151–1155
19 Dürr, A. *et al.* (1996) *N. Engl. J. Med.* 335, 1169–1175
20 Filla, A. *et al.* (1996) *Am. J. Hum. Genet.* 59, 554–560
21 Epplen, C. *et al.* (1997) *Hum. Genet.* 99, 834–836
22 Montermini, L. *et al.* (1997) *Hum. Mol. Genet.* 6, 1261–1266
23 Campuzano, V. *et al.* (1997) *Hum. Mol. Genet.* 6, 1771–1780
24 Koutnikova, H. *et al.* (1997) *Nat. Genet.* 16, 345–351
25 Gibson, T.J. *et al.* (1996) *Trends Neurosci.* 19, 465–468
26 Priller, J. *et al.* (1997) *Ann. Neurol.* 42, 265–269
27 Babcock, M. *et al.* (1997) *Science* 276, 1709–1712
28 Wilson, R.B. and Roof, D.M. (1997) *Nat. Genet.* 16, 352–357
29 Foury, F. and Cazzalini, O. (1997) *FEBS Lett.* 411, 373–377
30 Barbeau, A. (1980) *Can. J. Neurol. Sci.* 7, 455–468
31 Rötig, A. *et al.* (1997) *Nat. Genet.* 17, 215–217
32 Ben Hamida, M. *et al.* (1993) *Neurology* 43, 2179–2183
33 Klockgether, T. *et al.* (1996) *Lancet* 348, 830
34 Maciel, P. *et al.* (1995) *Am. J. Hum. Genet.* 57, 54–61
35 Robitaille, Y. *et al.* (1997) *Brain Pathol.* 7, 901–926
36 Chung, M.Y. *et al.* (1993) *Nat. Genet.* 5, 254–258
37 Chong, S.S. *et al.* (1995) *Nat. Genet.* 10, 344–350
38 Ludwig, A., Flockerzi, V. and Hofmann, F. (1997) *J. Neurosci.* 17, 1339–1349
39 Matsumura, R. *et al.* (1997) *Neurology* 49, 1238–1243
40 Stevanin, G. *et al.* (1997) *Neurology* 49, 1243–1246
41 Ikeuchi, T. *et al.* (1997) *Ann. Neurol.* 42, 879–884
42 Gomez, C.M. *et al.* (1997) *Ann. Neurol.* 42, 933–950
43 Riess, O. *et al.* (1997) *Hum. Mol. Genet.* 6, 1289–1293
44 Matsuyama, Z. *et al.* (1997) *Hum. Mol. Genet.* 6, 1283–1287
45 Quigley, C.A. *et al.* (1992) *J. Clin. Endocrinol. Metab.* 74, 927–933
46 Gusella, J.F. *et al.* (1985) *Nature* 318, 75–78
47 Duyao, M.P. *et al.* (1995) *Science* 269, 407–410
48 Skinner, P.J. *et al.* (1997) *Nature* 389, 971–974
49 Paulson, H.L. *et al.* (1997) *Neuron* 19, 333–344
50 Davies, S.W. *et al.* (1997) *Cell* 90, 537–548
51 Ikeda, H. *et al.* (1996) *Nat. Genet.* 13, 196–202
52 Matilla, A. *et al.* (1997) *Nature* 389, 974–978
53 Brunt, E.R. and van-Weerden, T.W. (1990) *Brain* 113, 1361–1382
54 Adelman, J.P. *et al.* (1995) *Neuron* 15, 1449–1454
55 Yue, Q. *et al.* (1997) *Am. J. Hum. Genet.* 61, 1078–1087
56 Baloh, R.W. *et al.* (1997) *Ann. Neurol.* 41, 8–16
57 Jodice, C. *et al.* (1997) *Hum. Mol. Genet.* 6, 1973–1978
58 Geschwind, D.H. *et al.* (1997) *Neurology* 49, 1247–1251

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Review

Mapping and chromosome analysis: the potential of fluorescence in situ hybridization

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Abstract

Fluorescence in situ hybridization (FISH) is a method widely used for the delineation of chromosomal DNA. FISH is applied in many areas of basic research as well as in clinical cytogenetics. In this review important technical improvements as well as the various applications of this method are summarized. In the first part different labeling and detection procedures are described and the potential of various kinds of probes are discussed. Recent developments in optical instrumentation and digital imaging procedures are outlined in the second part. The following important applications of FISH are discussed: (a) new strategies for high resolution mapping of DNA sequences; (b) detection of chromosomal aberrations in clinical material; (c) techniques allowing the simultaneous detection of numerous probes by multiple color FISH; and (d) the new approach of comparative genomic hybridization, allowing a rapid and comprehensive analysis of chromosomal imbalances in cell populations, which is particularly useful for the cytogenetic analysis of tumor samples.

Key words: Fluorescence in situ hybridization; Digital image microscopy; Multicolor FISH; Chromosomal mapping; Interphase cytogenetics; Comparative genomic hybridization

1. Introduction

In 1969, Gall and Pardue described the hybridization of radioactively labeled rRNA to tissue squashes allowing the in situ visualization of the complementary sequences, the rDNA, within cells (Gall and Pardue, 1969). Since then, in situ hybridization has been further developed and widely used for the detection of DNA or RNA sequences. For about two decades, delineation of specific chromosomal sequences was performed using radioactively labeled probes. However, since the development of procedures for non-isotopic labeling of nucleic acids in the early 1980s, non-radioactive in situ hybridization has become increasingly popular. The reasons accounting for this development, such as increase in speed, sensitivity, signal resolution and long-term storage of labeled probe, has been reviewed multifold (see, e.g., Raap et al., 1990; Lichter and Ward, 1990c; Lichter et al., 1991; McNeil et al., 1991; Trask, 1991). In this article we focus on the potential of fluorescence in situ hybridization, also referred to as FISH, which has become of particular importance for the simultaneous visualization of multiple target sequences. New developments in probe generation and labeling, in the detection of hy-

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bridized probes, as well as in instrumentation and digital imaging microscopy are described. The further development of FISH resulted in new approaches for chromosome analysis. In this paper we review the recent advancements in gene mapping as well as in the diagnosis of chromosomal changes. Special emphasis is given to a new approach called comparative genomic hybridization, which allows a comprehensive analysis of chromosomal imbalances.

2. Fluorescence in situ hybridization to chromosomal DNA

In situ hybridization represents a cytochemical technique which allows the specific detection of single nucleic acid sequences in morphologically preserved biological specimens. In principle this detection is based on the specific base pairing of two complementary nucleic acid sequences, the probe and the target sequences. Hybridized probes are detected via fluorochromes using epifluorescence microscopy, via colorimetric enzyme assays by transmission light microscopy, or via metallic compounds in the electron microscope. Note that some enzyme-mediated colored precipitates can be modified by metallic reagents to suite for analysis in the electron microscope.

DNA probes for FISH can be labeled directly, i.e., conjugated with fluorochromes, or indirectly by modifications introducing reporter groups (e.g., biotin) in the probe molecules. In the latter case detection of the probe is achieved via reporter-binding molecules (e.g., avidin) conjugated with fluorochromes, enzymes or with metallic compounds. The probe labeling systems currently available are summarized in recently published review articles (Raap et al., 1990; Lichter et al., 1991). Colorimetric assays may be preferred for routine diagnosis since storage for long periods of time is possible. However, FISH is the method of choice for many applications, since it allows to simultaneously visualize several target regions using spectrally distinct fluorochromes (see Table 1). It seems important to note that detection of directly labeled nucleic acid probes is in general faster and results in lower background, since the incubation of reporter-binding molecules is omitted. However, indirect labeling may yield in higher detection sensitivities, since more fluorochromes are bound to the probe. A wide variety of modified nucleotides for the direct and indirect labeling of nucleic acids are commercially available (see also Wiegant et al., 1991). Amplification of fluorescent signals can be performed using antibodies which are directed against the reporter-binding molecules (e.g., against avidin; Pinkel et al., 1986) or the fluorochromes themselves.

Table 1
List of fluorochromes used as conjugates for probe detection or as chromosomal counterstain *

Color	Conjugate	Counterstain
Blue	AMCA	DAPI
		Hoechst 33258
Green	FITC	Quinacrine
	Bidopy	
Red	Rhodamines	Propidium iodide
	Texas Red	
	Cy 3	
Far red	Cy 5	
Infra red	Cy 7	

* Note, that a chromosomal counterstain should be selected in a different color than the fluorochromes used for probe detection.

Fluorochromes suitable for the detection of hybridized probes should meet several requirements including: (a) they should yield high quantum efficiency; (b) it should be possible to conjugate them covalently to reporter binding molecules such as avidin or antibodies; (c) they should be small enough to allow penetration into cellular preparations; and (d) they should be stable in order to allow evaluation for longer time periods and quantitative measurements, etc. Recently, new classes of fluorochromes, such as cyanines Cy3, Cy5, etc., became commercially available. Since these are more stable than many of the commonly used dyes, they are likely to become important reagents for FISH.

In many kinds of specimen, autofluorescence interferes with detection of fluorescent signals. In order to circumvent this problem, fluorochromes are needed which can be differentiated from the autofluorescent dyes. Autofluorescence generally occurs at shorter wavelengths of the visible light.

In these cases differentiation can be achieved by the use of dyes emitting at longer wavelengths. Therefore, the development of the new cyanine dyes emitting in the far red (Cy5) or infrared (Cy7) range provide new tools, which are of advantage when using certain autofluorescent specimens. However, it should be noted that the optical detection system must be sensitive for infrared light (i.e., CCD cameras, see below).

Since autofluorescence often occurs in spectral ranges overlapping with the emission of commonly used fluorochromes, current activities in the development of new fluorochromes focus on the differentiation by time-resolved detection (Berverloo et al., 1990, 1992; Marriott et al., 1991; Seveus et al., 1992). This is achieved by the measurement of the fluorescence at certain times after pulsed excitation. Conventional fluorochromes and most substances contributing to autofluorescence emit for a time period which is in the range of nanoseconds. Thus, this fluorescence can be measured a few nanoseconds after excitation. Delayed fluorescent dyes show prolonged emission and thus can be distinguished by detecting, e.g., micro- or milliseconds after the pulsed excitation. The instrumentation for such an approach has already been developed. However, suitable fluorochromes exhibiting delayed luminescence are still an area of active research, since they must also meet the requirements listed above, i.e., for example they should be small enough for penetration, and it should be possible to bind them specifically to a probe.

3. Probes for FISH

A number of different types of probes for fluorescence in situ hybridization can be distinguished on the basis of the complexities of probe or target sequences: the alphoid and satellite probes detecting repeated targets; individual probes such as plasmid-, phage-, cosmid- or YAC-clones detecting single copy sequences of one locus; or composite probes allowing a specific painting (termed by Pinkel et al., 1988) of individual chromosomes or chromosomal regions.

Complex probes frequently contain interspersed repetitive sequences (IRS) like the long or short interspersed elements (LINEs and SINEs, respectively). Because these sequences would result in high hybridization background, unlabeled competitor DNA is hybridized to the probe in order to saturate the repetitive probe sequences (Landegent et al., 1987; Lichter et al., 1988a; Pinkel et al., 1988). This procedure has also been termed chromosomal in situ suppression (CISS) hybridization (Lichter et al., 1988a). An ideal source of competitor DNA is the Cot-1 fraction of genomic DNA containing the highly repetitive sequences.

In addition to the application of conventional cloning strategies various PCR protocols have been developed for the generation of probes for FISH. Universal PCR is applied where it is intended to amplify as many sites as possible from the template DNA. This can be achieved in several ways. One possibility is to allow annealing of the primer at multiple sites of the template by lowering the conditions of the hybridization stringency. This strategy has been called 'priming authorizing random mismatches (PARM-PCR)' (Milan et al., 1993). Another strategy is to use degenerated primers that are able to anneal at multiple sites of the template DNA. Telenius et al. (1992a) constructed a 22 nt 'DOP'-primer (degenerate oligonucleotide primer), harboring 6 variable nucleotides within its central part. Thus, there are 4^6 different primers in the reaction. During an initial amplification phase the annealing temperature is held very low (30°C) allowing binding of the specific hexamer at the 3′ end as well as the adjacent degenerate hexamer at frequent intervals (about every 10^4 bp). During a second phase the annealing temperature is increased to about 55°C for the specific annealing of the full 22 nt primer in order to amplify the products of the first PCR cycles (Telenius et al., 1992a). A similar approach using degenerated primers was described by Bohlander and coworkers (1992). Instead of Taq polymerase T7 DNA polymerase was used for the initial amplification steps at low annealing temperatures. According to these authors, T7 polymerase leads to longer stretches of newly synthesized DNA because of better strand displacement synthesis ca-

pabilities (Bohlander et al., 1992). Universal amplification is also obtained by 'linker-adapter-PCR', which is particularly suited for small sized template fragments. Sized DNA fragments are ligated to a linker-adapter oligonucleotide, and a primer directed against this adapter element can then be used for PCR amplification (Lüdecke et al., 1989; Chang et al., 1992; Vooijs et al., 1993).

Species-specific amplification of mammalian DNA can be achieved by IRS-PCR (interspersed repetitive sequence-PCR) (Nelson et al., 1989; Ledbetter et al., 1990) amplifying sequences between repeated elements. In principal this is achieved by using primers directed against short or long interspersed sequences (Alu- or LINE-1 elements). At least one member of a primer pair recognizes a species specific subsequence, thus allowing specific amplification of sequences from this species that are present in a different genetic background (e.g., DNA in hybrid cells, YACs in yeast cells, etc.). Optimization of primer sequences (e.g., for minimal self-complementation) is referenced below (see also Nelson et al., 1989; Brooks-Wilson et al., 1990; Cotter et al., 1990; Sinke et al., 1992).

In the following we briefly review the various kinds of probes currently used in FISH. For the detection of numerical chromosomal aberrations probes recognizing chromosome specific tandemly repeated sequences, e.g., members of the alphoid repeat family, have been widely used (for review see Willard and Waye, 1987; Lichter et al., 1991; Tkachuk et al., 1991). The basic unit of the alphoid sequences is a 171 basepair monomer which is organized in reiterated units of higher order. The monomer sequences are modified multifold and some of these modifications result in chromosome specific subsequences. Usually a repetitive probe consists of a single cloned DNA fragment containing one or only a few of the repeated elements. Alternative approaches for the generation of alphoid probes use the PCR technique to amplify chromosome specific subsequences from various sources of genomic DNA (Koch et al., 1989; Weier et al., 1990; Dunham et al., 1991). Primers recognizing such subsequences can be used for amplifications from whole human genomic DNA. In cases where such sequences are not available, primers flanking the chromosome specific subsequences of the alphoid repeat can be used for PCR from the DNA of individual chromosomes present in somatic hybrid cells or fractions of sorted human chromosomes.

The potential of these chromosome specific repetitive probes for the approach of interphase cytogenetics (see below) is based mainly on two characteristics: (a) their high efficiency in visualizing the target sequences which is due to the size of the labeled genomic region, and (b) the fact that these regions consist of heterochromatin which is highly condensed resulting in focal signals in interphase cell nuclei. However, the analysis of patient cells from various diseases sometimes reveals cases where the centromeric heterochromatin is highly decondensed. This can hamper the enumeration of hybridization signals significantly (see Döhner et al., 1992). Similarily, somatic pairing of the centromeric region could result in a misinterpretation as a monosomy. A tissue specific pairing of chromosome 1 and 17 has been described by Arnoldus et al. (1989, 1991c).

Probe sets that specifically stain individual chromosomes were first obtained from DNA libraries derived from flow-sorted chromosomes which are also available through the American Type Culture Collection (Cremer et al., 1988; Lichter et al., 1988b; Pinkel et al., 1988). The first 'chromosome painting' probes consisted of libraries with high proportions of vector DNA frequently causing high background. The re-cloning of these DNA inserts from phage into plasmid vectors resulted in favourable ratios of insert to vector sequences (Fuscoe et al., 1989). Today, painting probes cloned in bluescribe vector are available for all human chromosomes (Collins et al., 1991). These 'pBS libraries' can also be obtained as labeled probes from commercial sources.

Chromosome specific probes using flow sorted chromosomes were obtained by PCR in several ways. Using a primer-pair that was directed against the flanking sequences of the Charon-4A vector and thus allowing the amplification of the insert sequences, it was possible to obtain chromosome specific painting probes from bacteriophage lamda libraries (Burde and Leary, 1992).

Small amounts of flow sorted chromosomes could also be directly amplified either by DOP-PCR (Carter et al., 1992; Telenius et al., 1992b), IRS-PCR (Suijkerbuijk et al., 1992) or linker-adapter-PCR (Chang et al., 1992; Vooijs et al., 1993), see above). In contrast to Alu-PCR, DOP-PCR allows the amplification from DNA of any species. Thus, painting probes from flow sorted pig chromosomes could also be obtained using this approach (Langford et al., 1992).

Ideal tools for the staining of specific chromosomal subregions, e.g., a chromosomal band, are DNA libraries generated from microdissected chromosomes. Directly cloned dissected fragments (Lengauer et al., 1991a) or sequences cloned following linker-adapter-PCR (Lüdecke et al., 1989; Lengauer et al., 1991b) have been successfully applied. Recently it was demonstrated, that microdissected material from small numbers of samples could be amplified by universal PCR and directly used for efficient delineation of the target region (Bohlander et al., 1992; Melzer et al., 1992).

Another source for chromosome painting probes are somatic hybrid cells containing only one chromosome from a particular species (Kievits et al., 1990). Despite the high proportion of rodent DNA within such a probe, the single chromosomes, that were retained within the hybrid cells result in a specific painting of the corresponding chromosomes on human metaphase spreads. In the same way mouse chromosomes could be specifically painted using mouse/hamster hybrid cell lines (Boyle et al., 1990). Applying IRS-PCR it was possible to rapidly obtain human chromosome-specific painting probes out of DNA of monochromosomal hybrid cell lines (Lengauer et al., 1990; Lichter et al., 1990a).

Plasmid-, phage-, cosmid- and YAC-clones are frequently used for the analysis of single loci. The capability of the various kinds of probes to specifically visualize their target sequences was reviewed previously (e.g., Lichter et al., 1991). Since the efficiency correlates with the size of the target region, YAC clones are particularly suitable probes. The use of the total DNA from a yeast clone containing a YAC may suffer from background problems because of the large percentage of labeled yeast genomic DNA within the probe. However, this can be avoided by isolation of the YAC following pulsed field gel electrophoresis. A very practical alternative is provided by the IRS-PCR technique (see above) for selective amplification of human sequences. However, the protocol used for specific amplification of human DNA out of human/rodent hybrid cell lines turned out not to be sufficient for generating suitable FISH probes from YACs. Therefore, new conditions had to be designed with special emphasis on primers which (a) minimize the Alu-content of inter-Alu PCR products; (b) recognize highly conserved regions of Alu elements; and (c) result in products independent of the orientation of flanking Alu-elements (Baldini et al., 1992; Breen et al., 1992; Lengauer et al., 1992; Tagle and Collins, 1992). It should be noted that, following this PCR strategy, representative amplification of human sequences is only guaranteed, when sufficient repeated elements are present within the cloned insert. YAC libraries exhibit high percentages (10–50%) of clones with non-contiguous, co-ligated insert sequences (for review see van Ommen, 1993). FISH is the method of choice to determine the degree of chimerism in a YAC library. If YAC probes generated by IRS-PCR are not representative, smaller co-ligated fragments might not be visible.

4. Optical instrumentation and digital imaging

The potential of FISH has been greatly increased by advancements in digital imaging microscopy. Digitized images not only facilitate the handling and storage of optical information but also allow more sophisticated image analyses applying appropriate computer software. In general, two different kinds of optical systems are used: powerful epifluorescence microscopes equipped with confocal laser scanning devices or sensitive cameras. Confocal laser scanning microscopy allows to generate stacks of optical sections from a fluorescently labeled specimen. Each section can be stored as digitized image and stacks of consecutive sections can be used for three-dimensional

reconstruction of the labeled objects applying appropriate 3D imaging software. In many cases the spatial relation of differentially labeled targets can be already assessed by acquiring an optical section of the plane defined by the location of the signals of interest. Distance measurements are then reduced to a two-dimensional analysis omitting the need for somewhat circumstantial three-dimensional reconstructions. Confocal microscopy generally allows to resolve signals with a resolution of ≥ 0.5 μm in the z-axis and ≥ 0.2 μm in the x- and y-axes (Jovin and Arndt-Jovin, 1989; Shotton, 1989). It is used for the analysis of the three-dimensional structure of chromosomes, subchromosomal regions or the suprachromosomal organisation within cell nuclei. Thus, nuclear architecture can be investigated by visualization of nuclear entities – either by in situ hybridization or by immunodetection – and the analysis of their spatial relation using confocal laser scanning microscopy (see for example Arndt-Jovin et al., 1990; van Dekken et al., 1990; Rawlins et al., 1991; Zirbel et al., 1993). It should be noted that an alternative approach to 3D microscopy is provided by using stacks of digitized images, which are obtained by using a sensitive camera connected to a conventional microscope. Whereas in the confocal microscope an additional aperture reduces the out of focus fluorescence physically, in such a set of images this reduction is achieved by applying sophisticated algorithms (Arndt-Jovin et al., 1985; Hiraoka et al., 1987) which are based on the comparison of consecutive images and their deconvolutions. Originally this approach has only be used by highly specialized laboratories, since the requirements for computer hardware were very high. However, recent developments in computer hardware and software allowed to reduce these requirements resulting in commercially available systems suited for a broader application.

High quality digitized images are obtained using sensitive camera systems. The most sensitive system to date is the so-called cooled CCD (charged coupled device) camera. Its capability to count photons allows imaging of very low light over a wide range of wavelengths. Since this is achieved over a broad dynamic range, images generated by cooled CCD cameras are particularly useful for the objective, quantitative measurement of fluorescence signals. This provides the basis for the comparison of fluorescence intensities by the calculation of their ratios (for applications see below). In order to obtain a mean ratio, multiple comparisons have to be performed. This requires careful standardization of the conditions of image acquisition, such as the adequate standardization of excitation, lenses, camera integration time and sensitivity of the photomultiplier. The conditions have to be adjusted in order to avoid saturation of the signal image. Furthermore, the differences in fluorochrome stability and bleaching characteristics can also influence the ratio measurement. Therefore, fluorochromes of similar stability should be chosen or measurements should be standardized accordingly.

Digitized images of objects stained by several fluorochromes can in principle be obtained in two ways: (a) by using cameras recording color information; or (b) by generating gray-scale images separatly for each fluorochrome followed by pseudocolorization and electronical overlay. Due to the high magnification required for chromosome analysis, this overlaying can be critically influenced by the so-called registration problem. For the image acquisition of each fluorochrome filter cubes are moved containing the excitation filter, the dichroic mirror and the emission filter. Slight differences in the position of the filters result in a shift of the images. Overlaying of shifted images could affect the spatial relation of two differentially labeled chromosomal regions dramatically. Therefore their relation could only be concluded from overlayed images, when images are re-adjusted. This can be accomplished on various levels: (a) by fine adjustment of filter positions within a device of several filter combinations; (b) by using filters free of wedges, which would cause a shift of light pathway; (c) by fixing a dichroic mirror and changing adjusted emission and/or excitation filters on a separate wheel which is moved by motorized devices; (d) by using contures of the object such as chromosome boundaries or background fluorescence for re-adjustment by software; (e) by an additional hy-

bridization with a control probe simultaneously, detected via all fluorochromes used in the experiment and software-mediated re-adjustment according to these signals; (f) as in (e) but using multicolor fluorescent beads. Also, for the measurement of signal distances one probe could be detected by, e.g., FITC, whereas a second one is detected via FITC as well as, e.g., rhodamine. The rhodamine allows signal assignment, but the distance between the two probes is measured only using the FITC image. Thus, no shifting could influence the distance analysis.

New filter types are developed which allow the simultaneous detection of two or more fluorochromes. They consist of a filter with two or more narrow bandpasses for excitation and a filter with corresponding bandpasses for emission and include a specialized dichroic mirror. Since multiple bandpass filters yield in the simultaneous visualization of several fluorochromes, a digitized image acquired using such a filter allows to measure the geometric relation of differently stained targets without the need for re-adjustment. Multi-bandpass filters can also be used to align images obtained with single bandpass filters. For example, a multi-bandpass image showing fluorochromes A and B simultaneously can be used as a reference image. Seperate overlaying of images from fluorochrome A and B to this reference, respectively, reveals the image shifts relative to the reference. Based on this information, images for fluorochromes A and B can then be overlaid and realigned precisely.

It should be noted, that the multi-bandpass filters in general lower the detection sensitivity because of reduced transmission as compared to single bandpass filter sets, and therefore, strong fluorescent signals are required in such applications. Recently, colored CCD cameras have become commercially available. These use a significant proportion of the camera picture elements to gain color information. Although this results in some decrease of sensitivity and resolution, such cameras are of high practical value in many applications of FISH. Since image overlay is omitted, colored CCD cameras are particularly useful for routine work. New developments of color CCD cameras invest in the use of multiple chips, each for one color, in order to avoid the need for camera picture elements within a single chip. This should allow in the future to reach sensitivities comparable to gray level camera systems. Object areas stained by two or more fluorochromes and detected using multi-bandpass filters are visible in a mixed color. Thus, mixtures of two colors at different ratios become visible by differences in the final mixed color (see below). However, current instruments for simultaneous color recording seem not suitable for advanced applications of ratio imaging (see below) and quantitative fluorescence microscopy.

5. Multicolor fluorescence in situ hybridization

The potential of FISH is greatly increased by the possibility to simultaneously visualize multiple targets in a single specimen. When each target is delineated by a different fluorochrome, the number of targets is limited by the fluorochromes that can be distinguished spectrally. Accordingly, with the advent of the coumarin AMCA emitting in the blue spectral range, a third probe could be detected individually in addition to a green (e.g., FITC) and red (e.g., rhodamine) stained probe (Nederlof et al., 1989a, b). By the combination of different DNA labeling/detection systems it is possible to increase the number of detectable targets (Nederlof et al., 1990). The labeling of seven targets by combination of three colors was recently reported (Ried et al., 1992a; Wiegant et al., 1993). Using selective optical filters, gray-scale images were obtained for each of the three fluorochromes. Comparison of these images, e.g., by overlaying, revealed targets detected by one fluorochrome or by combinations of two or three fluorochromes. Based on this analysis, separate gray-scale images were generated for every differentiated target (e.g., one image from red signals only, one from green signals only, a third one from red and green only, etc.). Finally, the seven different gray-scale images were pseudocolored and merged to result in an image, where the seven different probes are discriminated by color.

Probes can also be discriminated by combina-

tion of colors at different ratios (Nederlof et al., 1992; Du Manoir et al., 1993). Probes are labeled by two or more reporter molecules added at variable proportions to the labeling reaction ('ratio-labeling'). Measuring different ratios of the two fluorochromes FITC/TRITC allowed to clearly distinguish between three different probes to which a pseudocolor could be assigned: ratios of 1:1, 1:10 and 1:50 (Nederlof et al., 1992); or ratios 4:1, 1:1 and 1:4 (Du Manoir et al., 1993). Dauwerse et al., demonstrated, that for large stained areas (such as painted chromosomes) mixing of FITC and TRITC in multiple ratios allowed to distinguish 7 different targets by the differences in the resulting real color as it is visible through a dual bandpass filter. Finally, by a combination of combinatorial and ratio labeling 12 different targets could be visualized simultaneously in real colors (Dauwerse et al., 1992). Based on these data and due to the availability of further fluorochromes (see above) one can envisage the simultaneous detection of all 24 different human chromosomes in discrete colors. The development of multicolor in situ hybridization is particularly useful for many diagnostic applications of FISH (see below).

6. Rapid mapping of DNA sequences

DNA sequences can be mapped by fluorescence in situ hybridization with speed and precision (Lichter and Ward, 1990c; Lichter et al., 1991; McNeil et al., 1991; Trask, 1991; Lawrence et al., 1992; Korenberg et al., 1992). A fast approach to order large numbers of cosmids along chromosomes uses geometrical mapping coordinates. The distance between the probe and the telomere of the short arm of the chromosome (pter) is expressed as a fraction of the total length of the chromosome (FLpter, fractional length from pter; Lichter et al., 1990b). However, there are polymorphisms in chromosome length (Trask et al., 1989b) which might affect the FLpter values. Furthermore, differential condensation along a chromosome might result in higher variabilities of the FLpter values when chromosomes of different condensation stage are used. Therefore, it is recommended to perform this analysis with the same homolog and with chromosomes of similar length. The accuracy might be improved by measuring fractional lengths within smaller chromosomal regions, e.g., defined by the borders of chromosome bands (Lawrence et al., 1990).

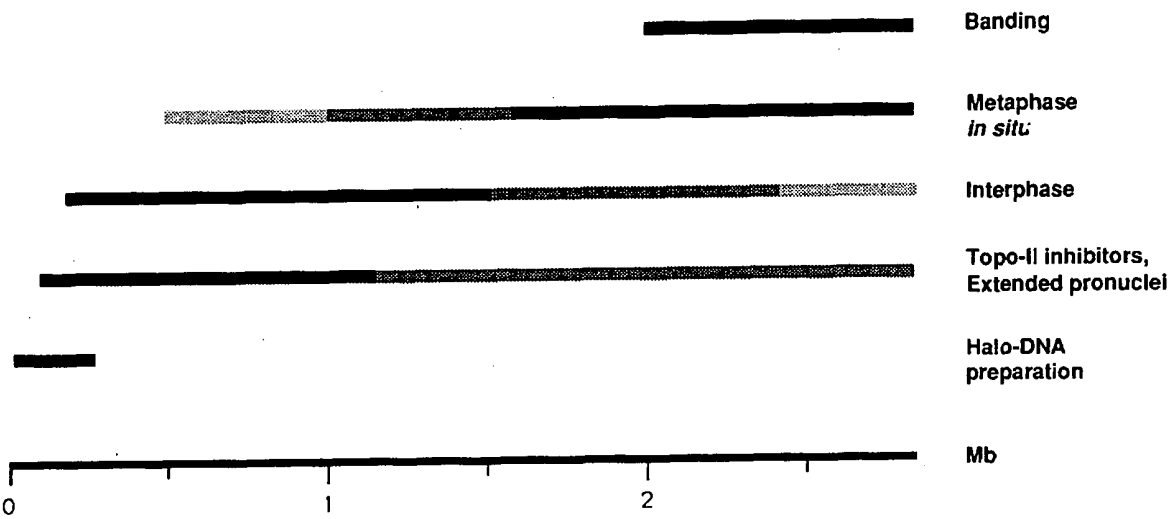


Fig. 1. Comparison of the mapping resolution achieved by using various molecular cytogenetic techniques. Established range of resolution is indicated in black, range of resolution depending on variabilities in preparations or packaging of DNA in certain genomic areas is indicated in gray.

For many applications it is necessary to relate a signal relative to chromosomal bands. Protocols for Giemsa banding prior to FISH experiments have been published (see, e.g., Klever et al., 1991). Whereas prehybridization banding is time-consuming since it requires the relocation of the same metaphases, protocols have been developed which allow a simultaneous visualization of bands and signals. Frequently this chromosome banding is achieved by DAPI (sometimes combined with actinomycin D), Hoechst 33258, chromomycin or quinacrine following in situ hybridization. Many of these banding procedures result in higher quality banding, when incorporation of the thymidine analogue BrdU and UV-irradiation is included. Successive staining with fluorochromes like DAPI, Hoechst 33258 or propidium iodide results in the generatation of R- or G-bands – depending on the stage of the cell cycle during BrdU application (Fan et al., 1990; Takahashi et al., 1990; Lemieux et al., 1992). An alternative method for simultaneous banding is based on the co-hybridization of differentially labeled, interspersed repetitive DNA probes. For example, human Alu sequences generate a R-band-like pattern that can be utilized for gene mapping analyses (Lichter et al., 1990b; Baldini and Ward, 1991).

Many analyses focus on the relative position of probes. The resolution of ordering two closely adjacent probes on chromosomes is obviously limited by the condensation state of the chromatin. The resolution limits on metaphase chromosomes might vary between laboratories, but a recent comprehensive study demonstrates, that probes separated by 1 Mb can still be ordered (Trask et al., 1993).

In order to increase the mapping resolution, preparations of chromatin with a lower state of DNA condensation has been utilized. The mapping resolutions achieved by these various techniques are summarized in Fig. 1. In order to obtain very elongated chromosomes, interphase cells can be fused with mitotic cells leading to 'premature chromosome condensation' (PCCs) (Rao and Johnson, 1970; Sperling and Rao, 1974). These structures are, in general, longer than metaphase chromosomes and can also be used for in situ hybridization (Evans et al., 1991). Standard PCC protocols use HeLa cells as the mitotic fusion partner. However, HeLa also contributes often to the PCC structures. Since HeLa contains a multifold rearranged genome, it is not suitable to use this cell line for PCC preparations in gene mapping. Alternatively, we have used rodent cells as mitotic fusion partners, but the yield of PCC preparations is in our hands not sufficient for broad scale mapping projects (our own unpublished observations).

An even higher resolution is achieved using preparations of interphase nuclei (Lawrence et al., 1988, 1990; Trask et al., 1989a, 1991b; van den Engh et al., 1992). Since the longitudinal extension of the chromatid cannot be visualized, ordering is achieved by indirect procedures. A set of probes is ordered by comparison of the average distances between pairs of probes labeled in the nucleus. It was shown, that there is a linear relationship between the distance of two probes on the DNA level and the average distance of the interphase hybridization signals measured. This linearity is observed within the range of 100 kb to 2000 kb distance (for references see above). Within this range the distances between hybridization signals in interphase nuclei follow a random walk model (van den Engh et al., 1992). Thus systematic influences due to higher order structures within the chromatin seem not to be important when using the experimental conditions applied in these studies (e.g., methanol/acetic acid fixation, flat nuclei, etc.). Further increase of the molecular distance does not allow unambiguous probe ordering. As an alternative to distance measurements, multiple probes labeled differentially can be hybridized to cell nuclei and probe ordering is performed by determining the highest frequence in which a certain order of, e.g., three probes occurs (for references see above).

More recently, the use of FISH to other chromatin preparation techniques allowed to further extend the mapping resolution. After in vitro fertilization of hamster eggs using hamster or human sperms, DNA from pronuclei can be prepared in form of highly decondensed chromatin fibers. Multicolor in situ hybridization to these fibers allows to order probes with a resolution

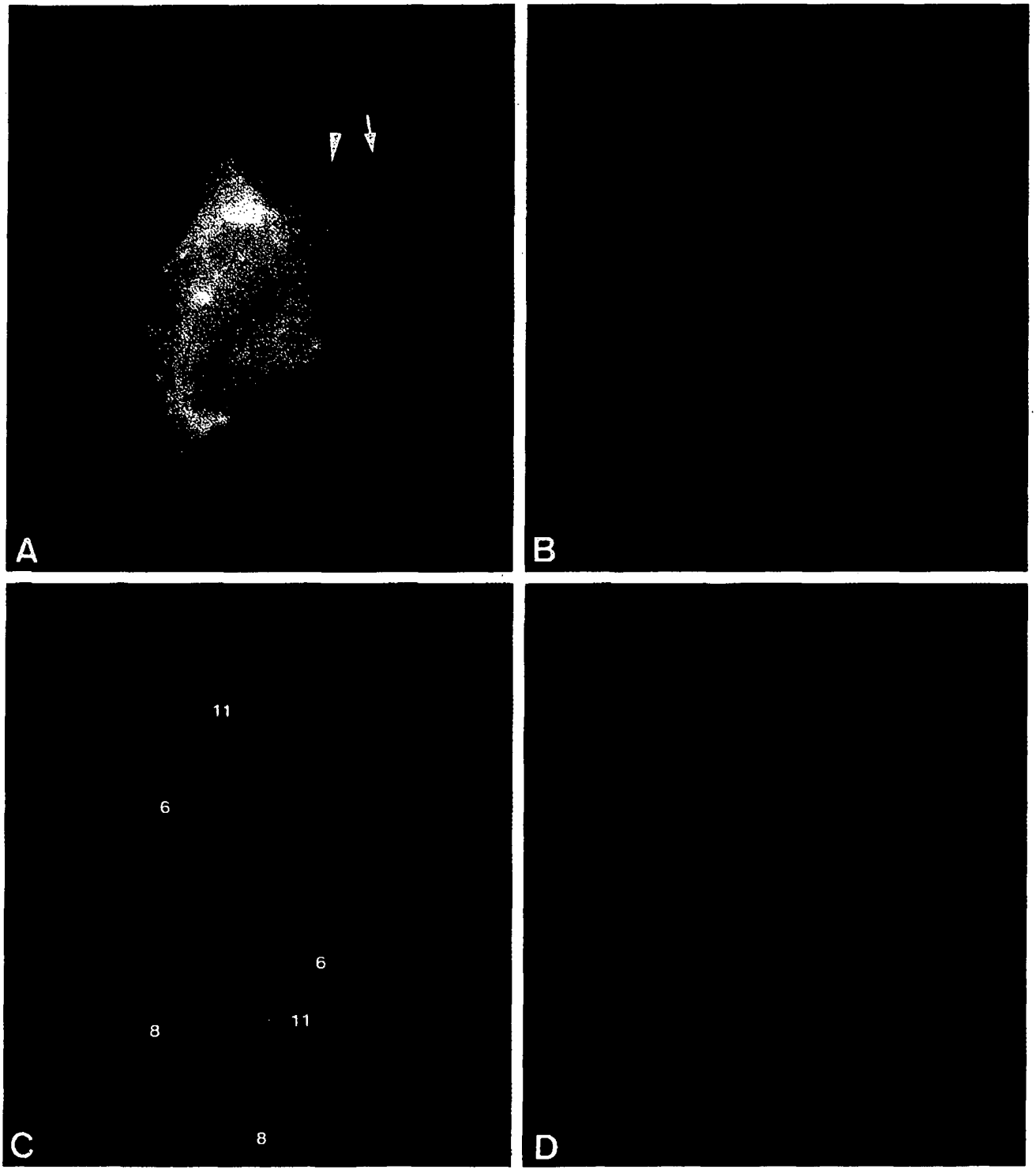
which is in the 20–800 kb range (Brandriff et al., 1991). A similar range has been reported for the use of so called 'free chromatin', which is prepared using inhibitors of chromatin condensation, in particular topoisomerase II inhibitors. On such structures probes separated between 21 and 300 kb could be mapped relative to each other (Heng et al., 1992).

Ultimately, very high resolution is achieved when FISH is applied to DNA halo preparations (Wiegant et al., 1992). High salt treatment of cell nuclei allows the preparation of highly extended DNA loops (Paulson and Laemmli, 1977; Vogelstein et al., 1980). Hybridization of single probes to the elongated DNA results in signals which appear like 'beads on a string'. Probes of, e.g., cosmid size label DNA which extends one to several μm in length (see Fig. 2A). Closely adjacent and even overlapping probes can be specifically delineated and ordered (Wiegant et al., 1992; Ried and co-workers, personal communication). Thus the maximal resolution is in the order of a few kb. On the other hand, probes further apart can only be ordered by this procedure when the DNA loop is visualized, e.g., by a co-hybridized probe set. Beyond the size of one DNA loop (60–200 kb) mapping seems not to be suitable. However, these structures can be used to answer questions about the specific localization of certain DNA sequences in DNA loops or at sites attached to the nuclear matrix (Lawrence et al., 1992, see also Fig. 2A).

7. Analysis of clinical materials by fluorescence in situ hybridization

Classical cytogenetics has contributed widely to the identification of genomic rearrangements found in genetic diseases and tumor cells. These rearrangements include numerical as well as structural chromosomal changes, such as deletions, duplications, inversions, translocations or amplifications. The latter might become visible by homogeneously staining regions (HSRs) or double minute (DM) chromosomes. By means of molecular genetics, genomic alterations are characterized on the molecular level pinpointing, e.g., chromosomal breakpoints to small DNA segments or even on the basepair level. Fluorescence in situ hybridization provides a link between chromosomal banding methods and such molecular analyses. A particular advantage of FISH is the potential to detect very small, submicroscopic chromosomal changes, such as small rearranged – e.g., translocated or deleted – material (see below). Furthermore in situ hybridization with suitable probes or probe sets allows to analyze chromosomal aberrations not only on metaphase chromosome spreads but also in interphase nuclei (see for example Fig. 2B). Since this analysis can be performed on the single cell level, it provides the basis for more detailed investigations of the clonal composition of cell populations in clinical samples. The applications of FISH to non-cycling cells allows a representative survey of the cell

Fig. 2. (A) Fluorescence in situ hybridization to the DNA halo preparation of a fibroblast nucleus. Two cosmid clones derived from the c-*myc* region on chromosome 8q24 known to be separated by about 200 kb were hybridized and detected with FITC and TRITC, respectively (see arrows). Chromatin is counterstained with DAPI-the nucleus is in the center (dense DAPI stain) and the halo-DNA is visible in the periphery (dimmer DAPI stain). Note the extension of the cosmid signals. This experiment is part of a study designed to analyze possible specific binding of DNA sequences to the nuclear matrix contained within the nucleus. (B) Example of an interphase cytogenetic analysis regarding the copy number of the RB-1 tumor supressor gene in cells of a patient suffering from a B-cell chronic lymphoid leukemia (B-CLL). Whereas in the metaphase spread two RB-1 homologs are visible, only one signal can be detected in the interphase nucleus indicating that the metaphase is most likely not derived from a leukemic cell, but possibly from a co-stimulated T-cell. This experiment is part of a study designed to detect the real frequency of certain chromosomal aberrations in the blood of B-CLL patients (see e.g., Stilgenbauer et al., 1993). (C and D) Comparative genomic hybridization (CGH) (for the procedure see scheme in Fig. 3 and text) revealing chromosomal imbalances in a T-cell prolymphocytic leukemia (T-PLL). DNA from normal cells was labeled with biotin and detected via FITC (c), whereas the tumor DNA was labeled with digoxigenin and detected via rhodamine (d). Deletions of chromosomal arms or smaller regions of it are clearly visible based on the weaker staining by the tumor DNA. For example see 6q, 8p, 11qter. Overrepresented chromosomal material can be identified by a stronger staining of chromosomal regions (see, e.g., 6p and 8q). An extensive analysis of this case has been published in Du Manoir et al. (1993).


A
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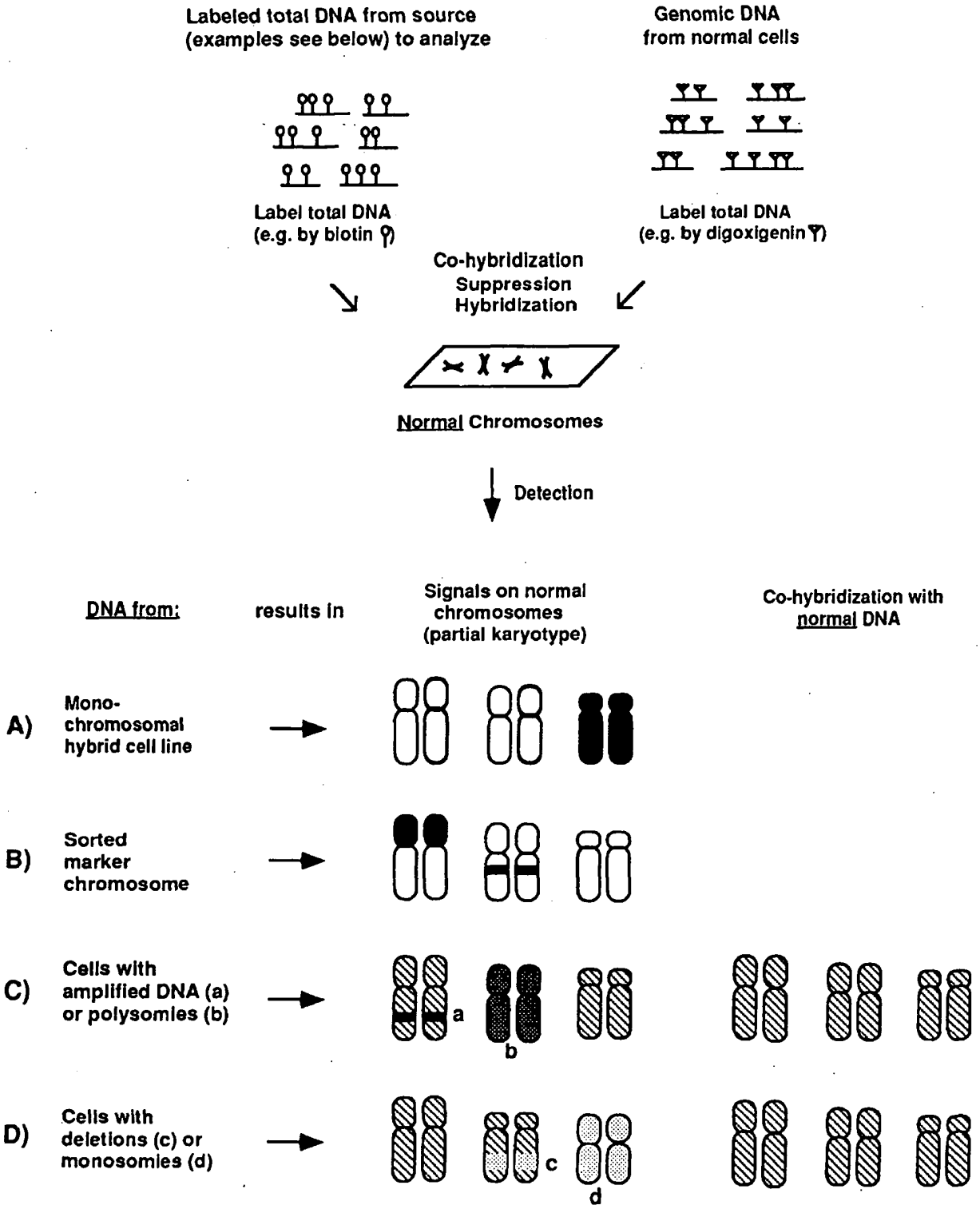
Labeled total DNA from source
(examples see below) to analyze
Genomic DNA
from normal cells
Label total DNA
(e.g. by biotin)
Label total DNA
(e.g. by digoxigenin)
Co-hybridization
Suppression
Hybridization
Normal Chromosomes
Detection
DNA from:
results in
Signals on normal
chromosomes
(partial karyotype)
Co-hybridization with
normal DNA
A)
Mono-
chromosomal
hybrid cell line
B)
Sorted
marker
chromosome
C)
Cells with
amplified DNA (a)
or polysomies (b)
a
b
D)
Cells with
deletions (c) or
monosomies (d)
c
d

population, which might be biased by selective mitogenic stimulation of subclones in metaphase analysis (Nederlof et al., 1989b; Du Manoir et al., 1993).

Analysis of numerical chromosomal changes in interphase nuclei was first performed on sex chromosomes by staining of the Barr- and Y-bodies (Barr and Bertram, 1949; Pearson et al., 1970). In situ hybridization of chromosome specific probes was used for the analysis of aneuploidies in spermatozoa nuclei (Joseph et al., 1984) and in amniotic fluid cells (Cremer et al., 1986). Since then, this approach is termed 'interphase cytogenetics' (Cremer et al., 1986). It is of particular advantage in cases where metaphase chromosomes cannot (or only hardly) be prepared, as, e.g., in many solid tumors. Interphase cytogenetics also facilitates the diagnosis of human amniotic fluid cells, chorionic villi samples (Collins et al., 1991; Chumakov et al., 1992; Klinger et al., 1992), blood or bone marrow smears (see, e.g., Anastasi et al., 1991; Bentz et al., 1993), and even of tissue section material (Manuelidis, 1985; Burns et al., 1986; Hopman et al., 1988; Emmerich et al., 1989; Arnoldus et al., 1991b; Rawlins et al., 1991; Hopman et al., 1992; Scherthan and Cremer, 1993; Stock et al., 1993). In general, this approach allows the evaluation of larger numbers of cells leading to more representative data collections. An alternative approach uses nuclei isolated from thick tissue sections which are spread on slides (Arnoldus et al., 1991a; Hopman et al., 1992). Whereas some of the morphological information is lost, this procedure allows sensitive delineation even of small target sequences.

Limitations of interphase cytogenetics are discussed in detail elsewhere (Tkachuk et al., 1991; Lichter and Ried, 1994). Large signals – as, e.g., whole chromosome paintings – are more likely to overlap in interphase nuclei. Therefore, more focal signals, for example obtained with most YACs or cosmids, are generally preferred. The hybridization efficiency of diagnostic probes must be very high, in particular, when mutiple probes are co-hybridized. For example, when each probe visualizes its target with an efficiency of 90%, the chance to detect three signals simultaneously will decrease below 75% ($0.9^3 = 0.729$). For more extensive discussions see Lengauer et al. (1993). An important application of multicolor FISH and interphase cytogenetics in prenatal diagnosis was recently demonstrated by Ried and colleagues (1992b).

A novel technique (MAC: morphology, antibody, chromosomes) that was initially described by Knuutila and co-workers allows to combine histological staining with labeling procedures by using antibodies and in situ hybridization (Knuutila and Teerenhovi, 1989; Larramedy and Knuutila, 1990; Tiainen et al., 1992; Kibbelaar et al., 1992). By this approach specific chromosomal aberrations can be related to certain cell types

Fig. 3. Schematic illustration of 'comparative genomic hybridization' (CGH) for chromosome analysis. Total DNA from cells to be analyzed ('test DNA', e.g., from tumor material) is labeled and hybridized to *normal* metaphase chromosomes. As an internal control differentially labeled DNA from normal cells ('control DNA') is co-hybridized with the test DNA under conditions of supression hybridization. The two DNAs are detected by different fluorochromes. Applications of this approach are indicated in the lower part of the scheme. (A) Hybridization of test DNA isolated from a mono-chromosomal hybrid cell line containing only a single human chromosome results in specific painting (indicated in black) of the corresponding chromosome in human metaphase spreads. The non-human chromosomes within this hybrid cell line do not contribute to a staining of the human chromosomes. (B) Using DNA of marker chromosomes as probe results in specific painting of the regions of which the marker is composed. In this way reverse painting allows the analysis of the origin of the marker. (C) Overrepresented sequences like amplifications (a) or polysomies (b) can be identified after hybridization with the test DNA. They result in a staining of the corresponding chromosomes or chromosomal regions (indicated in black), which is stronger than the general chromosomal labeling. Comparison with the control DNA allows a more reliable assessment of fluorescent intensities which are of diagnostic value, since variabilities in signal intensities can also be due to experimental parameters or DNA polymorphisms. (D) Partial or total loss of chromosomes (deletions (c) or monosomies (d)) can be identified by a weaker staining of the corresponding chromosomal regions (indicated in light grey). Again comparison with the control DNA allows a more reliable assessment of fluorescent intensities which are of diagnostic value.

and cell lineages. Thus, the potential for the diagnosis of tumor associated chromosomal alterations is extended considerably.

The use of multiprobe/multicolor FISH allows the development of chromosomal banding patterns that fit the needs of an experimental situation through the choice of an ordered set of clones for a given chromosome or chromosomal region (Cremer et al., 1986; Lichter et al., 1990b; Nederlof et al., 1990; Pinkel, D., Rutovitz, D., Gray, J., personal communication). Multiprobe FISH experiments defining each chromosome on a normal human karyotype were recently reported (Lengauer et al., 1993). Individual staining patterns are obtained by the order, distance and color of hybridization signals from probes or nested probe sets. In analogy to other digital identification procedures, the signals were termed 'bars' and the individual staining patterns 'chromosomal bar codes' (Lengauer et al., 1993). This approach is aimed to allow diagnosis of chromosomal aberrations by one or only a few hybridization experiments also providing the basis for automated chromosome analysis. It should be noted, that the more probes are co-hybridized the more the limitations regarding the efficiency in simultaneous probe detection apply (see above).

By in situ hybridization with increasing numbers of probes more chromosomal areas can be analyzed with regard to genomic alterations. Alternatively, a new and very powerful approach, which has recently been introduced, allows a comprehensive analysis of chromosomal imbalances (Kallioniemi et al., 1992). This method, which is outlined in Fig. 3, is based on a principle previously applied for the analysis of selected chromosomes and termed 'reverse chromosome painting' (Carter et al., 1992). Hybridization of the whole genomic DNA from an interspecies somatic cell hybrid to normal chromosomes from one parental species results in the delineation of species specific chromosomal regions (Boyle et al., 1990; Kievits et al., 1990). Similarily, the DNA of flow sorted human marker chromosomes can be hybridized to normal human chromosomes delineating the composition of the marker (Chang et al., 1992; Suijkerbuijk et al., 1992; Telenius et al., 1992b). By using whole genomic DNA from a certain cell population of one organism ('test DNA'), hybridization to normal chromosomes results in a more or less even labeling of the chromosomes. Over- or underrepresented chromosomal regions, however, result in a stronger or weaker staining, respectively, of the corresponding target sequences (see also Joos et al., 1993; Kallioniemi et al., 1993). Since signal variations also occur due to experimental parameters or polymorphic sequences, differentially labeled normal DNA ('control DNA') is co-hybridized and detected by a different fluorochrome (Fig. 3). Comparison of the signals generated by test and control DNA allows a more accurate assessment of chromosomal gains and losses. Hence, this appraoach was termed 'Comparative Genomic Hybridization (CGH)' (Kallioniemi et al., 1992). An example for the analysis by CGH is shown in Fig. 2B and C. Many chromosomal imbalances – in particular amplifications – are visible even without co-hybridization of control DNA (Kallioniemi et al., 1992; Joos et al., 1993). However, for a more sophisticated analysis, e.g., if the degree of over- or underrepresentation is to be analyzed, ratio comparisons as performed for CGH are the method of choice (Kallioniemi et al., 1992; Du Manoir et al., 1993). This comparison is based on high quality gray level images of each fluorochrome, acquired, e.g., using a cooled CCD camera (see above), and appropriate image analysis software for the comparison of signal intensities along the longitudinal axis of the chromosomes. At present, the identification of complete or partial monosomies, trisomies and (with a certain probability) tetrasomies by ratio measurements was reported (Kallioniemi et al., 1992; Du Manoir et al., 1993). Future developments on fluorochromes, optical instrumentation and image analysis are likely to further increase the potential of CGH by allowing to also differentiate, e.g., pentasomies, etc., or to detect monosomies and other imbalances in a subset of the analyzed cell population. The latter is of particular importance for the investigation of many tumors, since the tissue samples to be analyzed are frequently comprised only in part of tumor cells harbouring genomic alterations. Whereas amplified sequences of $\geq$ 100 kb become immediately

visible, the minimal size of a detectable chromosomal region present in one or three copies still has to be determined. Kallioniemi and co-workers reported the identification of interstitial deletions of around 10 Mb. It can be expected, that higher resolutions will be achieved, for example when using preparations of more elongated chromosomes (see above).

Analysis of solid tumors by CGH is often limited by very small amounts of tumor tissue available. Representative amplification of small amounts of DNA by universal PCR techniques (see above) might help to overcome this difficulty. A recent study defines the parameters needed for a successful application of DOP-PCR in combination with CGH (Speicher et al., in preparation).

Cytogenetic analysis of many tumors has been hampered by the fact, that metaphase chromosomes could not be prepared, or that stimulation of tumor cells in culture may affect chromosomal stability. These problems are circumvented by CGH, since it only requires DNA prepared from a given tissue sample. Therefore, this approach can be considered as major breakthrough for tumor cytogenetics greatly increasing the potential of the FISH techniques. Although it does not allow to detect balanced translocations or very small imbalances, it provides a powerful tool for a first screening of tumor material with unknown chromosomal aberrations obviating the need of testing large numbers of probes for single loci. This screening will provide information about the genomic sequences to be further analyzed by means of, e.g., interphase cytogenetics, Southern blot analysis, PCR amplifications, etc., in order to allow diagnosis at the molecular level or to unravel new genomic regions critical for tumor development or progression.

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References

Anastasi, J., Vardiman, J.W., Rudinsky, R., Patel, M., Nachman, J., Rubin, C.M. and LeBeau, M.M. (1991) Direct correlation of cytogenetic findings with cell morphology using in situ hybridization: an analysis of suspicious cells in bone marrow specimens of two patients completing therapy for acute lymphoblastic leukemia. Blood 77, 2456–2462.

Arndt-Jovin, D.J., Robert-Nicoud, M., Kaufman, S.J. and Jovin, T.M. (1985) Fluorescence digital imaging microscopy in cell biology. Science 230, 247–256.

Arndt-Jovin, D.J., Robert-Nicaud, M. and Jovin, T.M. (1990) Probing DNA structure and function with a multiwavelength fluorescence confocal laser microscope. J. Microsc. 157, 131–139.

Arnoldus, E.P.J., Peters, A.C.B., Bots, G.T.A.M., Raap, A.K. and van der Ploeg, M. (1989) Somatic pairing of chromosome 1 centromeres in interphase nuclei of human cerebellum. Hum. Genet. 83, 231–234.

Arnoldus, E.P., Dreef, E.J., Nordermeer, I.A., Verheggen, M.M., Thierry, R.P., Peters, A.C., Cornelisse, C.J., Van der Ploeg, P. and Raap, A.K. (1991a) Feasibility of in situ hybridization with chromosome specific DNA probes on paraffin wax embedded tissue. J. Clin. Pathol. 44, 900–904.

Arnoldus, E.P.J., Noordermeer, I.A., Peters, A.C.B., Voormolen, J.H.C., Bots, G.T.A.M., Raap, A.K. and van der Ploeg, M. (1991b) Interphase cytogenetics of brain tumors. Genes, Chromosomes Cancer 3, 101–107.

Arnoldus, E.P.J., Peters, A.C.B., Bots, G.T.A.M., Raap, A.K. and Van der Ploeg, M. (1991c) Interphase cytogenetics reveals somatic pairing of chromosome 17 centromeres in normal human brain tissue, but no trisomy 7 or sex chromosome loss. Cytogenet. Cell Genet. 56, 214–216.

Baldini, A. and Ward, D.C. (1991) In situ hybridization banding of human chromosomes with Alu-PCR Products: a simultaneous karyotype for gene mapping studies. Genomics 9, 770–774.

Baldini, A., Ross, M., Nizetic, D., Vatcheva, R., Lindsay, E.A., Lehrach, H. and Siniscalco, M. (1992) Chromosomal assignment of human YAC clones by fluorescence in situ Hybridization: Use of single-yeast-colony PCR and multiple labeling. Genomics 14, 181–184.

Barr, M.L. and Bertram, E.G. (1949) A morphological distinction between neurons of the male and female, and the behaviour of the nuleolar satellite during accelerated nuleioprotein synthesis. Nature 163, 676–677.

Bentz, M., Schröder, M., Herz, M., Stilgenbauer, S., Lichter, P. and Döhner, H. (1993) Detection of trisomy 8 on blood smears using fluorescence in situ hybridization. Leukemia 7, 752–757.

Berverloo, H.B., van Schadewijk, A., van Gelderen-Boele, S. and Tanke, H.J. (1990) Inorganic phosphors as new luminescent labels for immunocytochemistry and time-resolved microscopy. Cytometry 11, 784–792.

Beverloo, H.B., van Schadewijk, A., Bonnet, J., van der Geest, R., Runia, R., Verwoerd, N.P., Vrolijk, J., Ploem, J.S. and

Tanke, H.J. (1992) Preparation and microscopic visualization of multicolor luminescent immunophosphors. Cytometry 13, 561–570.

Bohlander, S.K., Espinosa, R.3., Le, B.M., Rowley, J.D. and Diaz, M.O. (1992) A method for the rapid sequence-independent amplification of microdissected chromosomal material. Genomics 13, 1322–1324.

Boyle, A.L., Lichter, P. and Ward, D.C. (1990) Rapid analysis of mouse-hamster hybrid cell lines by in situ hybridization. Genomics 7, 127–130.

Brandriff, B., Gordon, L. and Trask, B. (1991) A new system for high-resolution DNA sequence mapping in interphase pronuclei. Genomics 10, 75–82.

Breen, M., Arveiler, B., Murray, I., Gosden, J.R. and Porteous, D.J. (1992) YAC mapping by FISH using Alu-PCR-generated probes. Genomics 13, 726–730.

Brooks-Wilson, A.R., Goodfellow, P.N., Povey, S., Nevanlinna, H.A., de Jong, P.J. and Goodfellow, P.J. (1990) Rapid cloning and characterization of new chromosome 10 DNA markers by Alu-element mediated PCR. Genomics 7, 614–620.

Burde, S. and Leary, J.F. (1992) Detection of individual human chromosomes by chromosome in situ suppression hybridization using PCR-amplified bacteriophage library probes. GATA 9, 64–67.

Burns, J., Redfern, D.R.M., Esiri, M.M. and McGee, J.O.D. (1986) Human and viral gene detection in routine paraffine embedded tissue by in situ hybridization with biotinylated probes: viral localization in herpes encephalitis. J. Clin. Pathol. 39, 1066–1073.

Carter, N.P., Ferguson, S.M., Perryman, M.T., Telenius, H., Pelmear, A.H., Leversha, M.A., Glancy, M.T., Wood, S.L., Cook, K., Dyson, H.M., et al. (1992) Reverse chromosome painting: a method for the rapid analysis of aberrant chromosomes in clinical cytogenetics. J. Med. Genet. 29, 299–307.

Chang, K.S., Vyas, R.C., Deaven, L.L., Trujillo, J.M., Stass, S.A. and Hittelman, W.N. (1992) PCR amplification of chromosome-specific DNA isolated from flow cytometry-sorted chromosomes. Genomics 12, 307–312.

Chumakov, I.M., Le Gall, I., Billaut, A., Ougen, P., Soularue, P., Guillou, S., Rigault, P., Bui, H., De Tant, Barillot, E., Abderrahim, H., Cherif, D., Berger, R., Le Paslier, D. and Cohen, D. (1992) Isolation of chromosome 21-specific yeast artificial chromosomes from a total human genome library. Nature Genet. 1, 222–225.

Collins, C., Kuo, W.L., Segraves, R., Fuscoe, J., Pinkel, D. and Gray, J.W. (1991) Construction and characterization of plasmid libraries enriched in sequences from single human chromosomes. Genomics 11, 997–1006.

Cotter, F.E., Hampton, G.M., Nasipuri, S., Bodmer, W.F. and Young, B.D. (1990) Rapid isolation of human chromosome-specific DNA probes from a somatic cell hybrid. Genomics 7, 257–263.

Cremer, T., Landegent, J., Brückner, A., Scholl, H.P., Schardin, M., Hager, H.D., Devilee, P., Pearson, P. and van der Ploeg, M. (1986) Detection of chromosome aberrations in the human interphase nucleus by visualization of specific target DNAs with radioactive and non-radioactive in situ hybridization techniques: diagnosis of trisomy 18 with probe L1.84. Hum. Genet. 74, 346–352.

Cremer, T., Lichter, P., Borden, J., Ward, D.C. and Manuelidis, L. (1988) Detection of chromosome aberrations in metaphase and interphase tumor cells by in situ hybridization using chromosome specific library probes. Hum. Genet. 80, 235–246.

Dauwerse, J.G., Wiegant, J., Raap, A.K., Breuning, M.H. and van Ommen, G.J.B. (1992) Multiple colors by fluorescence in situ hybridization using radio-labelled DNA probes create a molecular karyotype. Hum. Mol. Genet. 1, 593–598.

Döhner, H., Pohl, S., Bulgay-Mörschel, M., Stilgenbauer, S., Bentz, M. and Lichter, P. (1992) Trisomy 12 in chronic lymphoid leukemias – a metaphase and interphase cytogenetic analysis. Leukemia 7, 716–720.

Du Manoir, S., Speicher, M.R., Joos, S., Schröck, E., Popp, S., Döhner, H., Kovacs, G., Robert-Nicoud, M., Lichter, P. and Cremer, T. (1993) Detection of complete and partial chromosome gains and losses by comparative genomic in situ hybridiyzation. Hum. Genet. 90, 590–610.

Dunham, I., Lengauer, C., Cremer, T. and Featherstone, T. (1991) Rapid generation of chromosome specific alphoid DNA probes using the polymerase chain reaction. Hum. Genet. 88, 457–462.

Emmerich, P., Jauch, A., Hofmann, M.-C., Cremer, T. and Walt, H. (1989) Interphase cytogenetics in paraffin embedded sections from human testicular germ cell tumor xenografts and in corresponding cultured cells. Lab. Invest. 61, 235–242.

Evans, J.W., Chang, J.A., Giaccia, A.J., Pinkel, D. and Brown, J.M. (1991) The use of fluorescence in situ hybridization combined with premature chromosome condensation for the identification of chromosome damage. Br. J. Cancer 63, 517–521.

Fan, Y.-S., Davis, L.M. and Shows, T.B. (1990) Mapping small DNA sequences by fluorescence in situ hybridization directly on banded metaphase chromosomes. Proc. Natl. Acad. Sci. USA 87, 6223–6227.

Fuscoe, J.C., Collins, C.C., Pinkel, D. and Gray, J.W. (1989) An efficient method for selecting unique-sequence clones from DNA libraries and its application to fluorescent staining of human chromosome 21 using in situ hybridization. Genomics 5, 100–109.

Gall, J.G. and Pardue, M.L. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc. Natl. Acad. Sci. USA 63, 378–383.

Heng, H.H.Q., Squire, J. and Tsui, L.-C. (1992) High-resolution mapping of mammalian genes by in situ hybridization to free chromatin. Proc. Natl. Acad. Sci. USA 89, 9509–9513.

Hiraoka, Y., Sedat, J.W. and Agard, D.A. (1987) The use of a charge-coupled device for quantitative optical microscopy of biological structures. Science 238, 36–41.

Hopman, A.H.N., Ramaekers, F.C.S., Raap, A.K., Beck,

J.L.M., Devilee, P., van der Ploeg, M. and Vooijs, G.P. (1988) In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. Histochemistry 89, 307–316.

Hopman, A.H.N., Poddighe, P., Moesker, O. and Ramaekers, F.C.S. (1992) Interphase cytogenetics: an approach to the detection of genetic aberrations in tumours. Diagnostic Molecular Pathology. A practical approach. IRL Press Inc., Oxford, pp. 142–167.

Joos, S., Scherthan, H., Speicher, M.R., Schlegel, J., Cremer, T. and Lichter, P. (1993) Detection of amplified genomic sequences by reverse chromosome painting using genomic tumor DNA as probe. Hum. Genet. 90, 584–589.

Joseph, A.M., Gosden, J.R. and Chandley, A.C. (1984) Estimation of aneuploidy levels in human spermatozoa using chromosome specific probes and in situ hybridization. Hum. Genet. 66, 234–238.

Jovin, T.M. and Arndt-Jovin, D.J. (1989) Luminescence digital imaging microscopy. Annu. Rev. Biophys. Biophys. Chem. 18, 271–308.

Kallioniemi, A., Kallioniemi, O.-P., Sudar, D., Rutovitz, D., Gray, J.W., Waldman, F. and Pinkel, D. (1992) Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258, 818–821.

Kallioniemi, O.-P., Kallioniemi, A., Sudar, D., Rutowitz, D., Gray, J.W., Waldman, F. and Pinkel, D. (1993) Comparative genomic hybridization, a rapid new method for detecting and mapping DNA amplification in tumors. Cancer Biol. 4, 41–46.

Kibbelaar, R.E., van Kamp, H., Dreef, E.J., de Groot-Swings, G., Kluin-Nelemans, J.C., Beverstock, G.C., Fibbe, W.E. and Kluin, Ph.M. (1992) Combined immunophenotyping and DNA in situ hybridization to study lineage involvement in patients with myeloblastic syndromes. Blood 79, 1823–1828.

Kievits, T., Devilee, P., Wiegant, J., Wapenaar, M.C., Cornelisse, C.J., van Ommen, G.J.B. and Pearson, P.L. (1990) Direct nonradioactive in situ hybridization of somatic cell hybrid DNA to human lymphocyte chromosomes. Cytometry 11, 105–109.

Klever, M., Grond-Ginsbach, C., Scherthan, H. and Schroeder-Kurth, T.M. (1991) Chromosomal in situ suppression hybridization after Giemsa banding. Hum. Genet. 86, 484–486.

Klinger, K., Landes, G., Shook, D., Harvey, R., Lopez, L., Locke, P., Lerner, T., Osathanondh, R., Leverone, B., Houseal, T., et al. (1992) Rapid detection of chromosome aneuploidies in uncultured amniocytes by using fluorescence in situ hybridization (FISH). Am. J. Hum. Genet. 51, 55–65.

Knuutila, S. and Teerenhovi, L. (1989) Immunophenotyping of aneuploid cells. Cancer Genet. Cytogenet. 41, 1–17.

Koch, J.E., Kølvraa, S., Petersen, K.B., Gregersen, N. and Bolund, L. (1989) Oligonucleotide-priming methods for the chromosome-specific labelling of alpha satellite DNA in situ. Chromosoma 98, 259–265.

Korenberg, J.R., Vang-Feng, T., Schreck, R. and Chen, X.N. (1992) Using fluorescence in situ hybridization (FISH) in genome mapping. Trends Biotechnol. 10, 27–32.

Landegent, J.E., Jansen in de Wal, N., Dirks, R.W., Baas, F. and van der Ploeg, M. (1987) Use of whole cosmid cloned genomic sequences for chromosomal localization by non-radioactive in situ hybridization. Hum. Genet. 77, 366–370.

Langford, C.F., Telenius, H., Carter, N.P., Miller, N.G.A. and Tucker, E.M. (1992) Chromosome painting using chromosome-specific probes from flow-sorted pig chromosomes. Cytogenet. Cell Genet. 61, 221–223.

Larramedy, M. and Knuutila, S. (1990) Immunophenotype and sister chromatid differentiation: A combined methology for analysing cell proliferation in unfractionated lymphocyte cultures. Exp. Cell Res. 188, 209–213.

Lawrence, J.B., Villnave, C.A. and Singer, R.H. (1988) Interphase chromatin and chromosome gene mapping by fluorescence detection of in situ hybridization reveals the presence and orientation of two closely integrated copies of EBV in a human lymphoblastoid cell line. Cell 52, 51–61.

Lawrence, J.B., Singer, R.H. and McNeil, J.A. (1990) Interphase and metaphase resolution of different distances within the human dystrophin gene. Science 249, 928–932.

Lawrence, J.B., Carter, K.C. and Gerdes, M.J. (1992) Extending the capabilities of interphase chromatin mapping. Nature Genet. 2, 171–172.

Ledbetter, S.A., Garcia-Heras, J. and Ledbetter, D.H. (1990) 'PCR-Karyotype' of human chromosomes in somatic cell hybrids. Genomics 8, 614–622.

Lemieux, N., Dutrillaux, B. and Viegas, P.E. (1992) A simple method for simultaneous R- or G-banding and fluorescence in situ hybridization of small single-copy genes. Cytogenet. Cell Genet. 59, 311–312.

Lengauer, C., Riethman, H. and Cremer, T. (1990) Painting of human chromosomes generated from hybrid cell lines by PCR with Alu and L1 primers. Hum. Genet. 86, 1–6.

Lengauer, C., Eckelt, A., Weith, A., Endlich, N., Ponelies, N., Lichter, P., Greulich, K.O. and Cremer, T. (1991a) Painting of defined chromosomal regions by in situ suppression hybridization of libraries from laser-microdissected chromosomes. Cytogenet. Cell Genet. 56, 27–30.

Lengauer, C., Lüdecke, H.-J., Wienberg, J., Cremer, T. and Horsthemke, B. (1991b) Comparative chromosome band mapping in primates by in situ suppression of band specific DNA microlibraries. Hum. Evol. 6, 67–72.

Lengauer, C., Green, E.D. and Cremer, T. (1992) Fluorescence in situ hybridization of YAC clones after Alu-PCR amplification. Genomics 13, 826–828.

Lengauer, C., Speicher, M.R., Popp, S., Jauch, A., Taniwaki, M., Nagaraja, R., Riethman, H.C., Donis-Keller, H., D'Urso, M., Schlessinger, D. and Cremer, T. (1993) Chromosomal bar codes produced by multicolor fluorescence in situ hybridization with multiple YAC clones and whole chromosome painting probes. Hum. Molec. Genet. 2, 505–512.

Lichter, P., Cremer, T., Borden, J., Manuelidis, L. and Ward, D.C. (1988a) Delineation of individual human chromo-

somes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. Hum. Genet. 80, 224–234.

Lichter, P., Cremer, T., Tang, C.C., Watkins, P.C., Manuelidis, L. and Ward, D.C. (1988b) Rapid detection of human chromosome 21 aberrations by in situ hybridization. Proc. Natl. Acad. Sci. USA 85, 9664–9668.

Lichter, P., Ledbetter, S.A., Ledbetter, D.H. and Ward, D.C. (1990a) Fluorescence in situ hybridization with Alu and L1 polymerase chain reaction probes for rapid characterization of human chromosomes in hybrid cell lines. Proc. Natl. Acad. Sci. USA 87, 6634–6638.

Lichter, P., Tang, C.C., Call, K., Hermanson, G., Evans, G.A., Housman, D. and Ward, D.C. (1990b) High resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. Science 247, 64–69.

Lichter, P. and Ward, D.C. (1990c) Is non-isotopic in situ hybridization finally coming of age? Nature 345, 93–95.

Lichter, P., Boyle, A.L., Cremer, T. and Ward, D.C. (1991) Analysis of genes and chromosomes by non-isotopic in situ hybridization. Genet. Anal. Technol. Appl. 8, 24–35.

Lichter, P. and Ried, T. (1994) Molecular analysis of chromosome aberrations: In situ hybridization. Methods in Molecular Biology: Chromosome Analysis Protocols. Humana Press, Clifton, NJ, pp. 449–478.

Lüdecke, H.J., Senger, G., Claussen, U. and Horsthemke, B. (1989) Cloning defined regions of the human genome by microdissection of banded chromosomes and enzymatic amplification. Nature 338, 348–350.

Manuelidis, L. (1985) In situ detection of DNA sequences using biotinylated probes. Focus 7, 4–8.

Marriott, G., Clegg, R.M., Arndt-Jovin, D.J. and Jovin, T.M. (1991) Time resolved imaging microscopy. Biophys. J. 60, 1374–1387.

McNeil, J.A., Johnson, C.V., Carter, K.C., Singer, R.H. and Lawrence, J.B. (1991) Localizing DNA and RNA within nuclei and chromosomes by fluorescence in situ hybridization. Genet. Anal. Technol. Appl. 8, 41–58.

Melzer, P.S., Guan, X.Y., Burgess, A. and Trent, J.M. (1992) Rapid generation of region specific probes by chromosome microdissection and their application. Nature Genet. 1, 24–28.

Milan, D., Yerle, M., Schmitz, A., Chaput, B., Vaiman, M., Frelat, G. and Gellin, J. (1993) A PCR-based method to amplify DNA with random primers: determining the chromosomal content of porcine flow-karyotype peaks by chromosome painting. Cytogenet. Cell. Genet. 62, 139–141.

Nederlof, P.M., Robinson, D., Abuknesha, R., Wiegant, J., Hopman, A.H.N., Tanke, H.J. and Raap, A.K. (1989a) Three-color fluorescence in situ hybridization for the simultaneous detection of multiple nucleic acid sequences. Cytometry 10, 20–27.

Nederlof, P.M., van der Flier, S., Raap, A.K., Tanke, H.J., van der Ploeg, M., Kornips, F. and Geraedts, J.P.M. (1989b) Detection of chromosome aberrations in interphase tumor nuclei by nonradioactive in situ hybridization. Cancer Genet. Cytogenet. 42, 87–98.

Nederlof, P.M., van der Flier, S., Wiegant, J., Raap, A.K., Tanke, H.J., Ploem, J.S. and van der Ploeg, M. (1990) Multiple fluorescence in situ hybridization. Cytometry 11, 126–131.

Nederlof, P.M., Flier van der, S., Vrolijk, J., Tanke, H.J. and Raap, A.K. (1992) Fluorescence ratio measurements of double-labeled probes for multiple in situ hybridization by digital imaging microscopy. Cytometry 13, 839–845.

Nelson, D.L., Ledbetter, S.A., Corbo, L., Victoria, M.F., Ramirez-Solis, R., Webster, T.D., Ledbetter, D.H. and Caskey, C.T. (1989) Alu polymerase chain reaction: a method for rapid isolation of human-specific sequences from complex DNA sources. Proc. Natl. Acad. Sci. USA 86, 6686–6690.

Paulson, J.R. and Laemmli, U.K. (1977) The structure of histone-depleted metaphase chromosomes. Cell 12, 817–828.

Pearson, P.L., Bobrow, M. and Vosa, C.G. (1970) Technique for identifying Y chromosomes in human interphase nuclei. Nature 226, 78–80.

Pinkel, D., Straume, T. and Gray, J.W. (1986) Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. Proc. Natl. Acad. Sci. USA 83, 2934–2938.

Pinkel, D., Landegent, J., Collins, C., Fuscoe, J., Segraves, R., Lucas, J. and Gray, J.W. (1988) Fluorescence in situ hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. Proc. Natl. Acad. Sci. USA 85, 9138–9142.

Raap, A.K., Dirks, R.W., Jiwa, N.M., Nederlof, P.M. and van der Ploeg, M. (1990) In situ Hybridization with Hapten-modified DNA Probes. Modern Pathology of AIDS and Other Retroviral Infections. S. Karger, Basel, pp. 17–28.

Rao, P.N. and Johnson, R.T. (1970) Mammalian cell fusion: Studies on the regulation of DNA synthesis. Nature 225, 159–164.

Rawlins, D.J., Highett, M.I. and Shaw, P.J. (1991) Localization of telomeres in plant interphase nuclei by in situ hybridization and 3D confocal microscopy. Chromosoma 100, 424–431.

Ried, T., Baldini, A., Rand, T.C. and Ward, D.C. (1992a) Simultaneous visualization of seven different DNA probes by in situ hybridization using combinatorial fluorescence and digital imaging microscopy. Proc. Natl. Acad. Sci. USA 89, 1388–1392.

Ried, T., Landes, G., Dackowski, W., Klinger, K. and Ward, D. (1992b) Multicolor fluorescence in situ hybridization for the simultaneous detection of probe sets for chromosome 13, 18, 21, X and Y in uncultured amniotic fluid cells. Hum. Molec. Genet. 1, 307–313.

Scherthan, H. and Cremer, T. (1994) Methology of non-isotopic in situ hybridization in paraffin-embedded tissue sections. Methods in Molecular Genetics. Academic Press, New York, in press.

Seveus, L., Väisälä, M., Syrjänen, S., Sandberg, M., Kuusisto, A., Harju, R., Salo, J., Hemmilä, I., Kojola, H. and Soini, E. (1992) Time-resolved fluorescence imaging of europium chelat label in immunochemistry and in situ hybridization. Cytometry 13, 329–338.

Shotton, D.M. (1989) Confocal scanning optical microscopy

and its applications for biological specimens. J. Cell Sci. 94, 175–206.

Sinke, R.J., Suijkerbuijk, R.F., Herbergs, J., Janssen, H., Cassiman, J.J. and Geurts van Kessel, A. (1992) Generation of a panel of somatic cell hybrids containing fragments of human chromosome 12p by X-ray irradiation and cell fusion. Genomics 12, 206–213.

Sperling, K. and Rao, P.N. (1974) The phenomenon of premature chromosome condensation: its relevance to basic and applied research. Humangenetik 23, 235–258.

Stilgenbauer, S., Döhner, H., Bulgay-Mörschel, M., Weitz, S., Bentz, M. and Lichter, P. (1993) High frequency of monoallelic retinoblastoma gene deletion in B-cell chronic lymphoid leukemia revealed by interphase cytogenetics. Blood 81, 2118–2124.

Stock, C., Ambros, I.M., Mann, G., Gadner, H., Amann, G. and Ambros, P.F. (1993) Detection of 1p36 deletions in paraffin sections of neuroblastoma tissues. Genes, Chromosomes Cancer 6, 1–9.

Suijkerbuijk, R.F., Matthopoulos, D., Kearney, L., Monard, S., Dhut, S., Cotter, F., Herbergs, J., van Kessel, A.G. and Young, B.D. (1992) Fluorescent in situ identification of human marker chromosomes using flow sorting and Alu element-mediated PCR. Genomics 13, 355–362.

Tagle, D.A. and Collins, F.S. (1992) An optimized Alu-PCR primer pair for human-specific amplification of YACs and somatic cell hybrids. Hum. Mol. Genet. 1, 121–122.

Takahashi, E.-i., Hori, T.-a., O'Connell, P., Leppert, M. and White, R. (1990) R-banding and nonisotopic in situ hybridization: precise localization of the human type II collagen gene (COL2A1). Hum. Genet. 86, 14–16.

Telenius, H., Carter, N.P., Bebb, C.E., Nordenskjöld, M., Ponder, B.A.J. and Tunnacliffe, A. (1992a) Degenerate oligonucleotide-primed PCR: General amplification of target DNA by a single degenerate primer. Genomics 13, 718–725.

Telenius, H., Pelmear, A.H., Tunnacliffe, A., Carter, N.P., Behmel, A., Ferguson-Smith, M.A., Nordenskjöld, M., Pfragner, R. and B.A.J., P. (1992b) Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. Genes, Chromosomes Cancer 4, 257–263.

Tiainen, M., Popp, S., Parlier, V., Emmerich, P., Bellomo, M.J., Ruutu, T., Cremer, T. and Knuutila, S. (1992) Chromosomal in situ suppression hybridization of immunologically classified mitotic cells in hematologic malignancies. Genes, Chromosomes Cancer 4, 135–140.

Tkachuk, D.C., Pinkel, D., Kuo, W.-L., Weier, H.-U. and Gray, J.W. (1991) Clinical applications of fluorescence in situ hybridization. Genet. Anal. Technol. Appl. 8, 67–74.

Trask, B., Pinkel, D. and van den Engh, G. (1989a) The proximity of DNA sequences in interphase cell nuclei is correlated to genomic distance and permits ordering of cosmids spanning 250 kilobase pairs. Genomics 5, 710–717.

Trask, B., van den Engh, G. and Gray, J.W. (1989b) Inheritance of chromosome heteromorphisms analyzed by high-resolution bivariate flow karyotyping. Am. J. Hum. Genet. 45, 753–760.

Trask, B. (1991a) Fluorescence in situ hybridization: applications in cytogenetics and gene mapping. TIG 7, 149–154.

Trask, B., Massa, H., Kenwrick, S. and Gitschier, J. (1991b) Mapping of human chromosome Xq28 by two-color fluorescence in situ hybridization of DNA sequences to interphase cell nuclei. Am. J. Hum. Genet. 48, 1–15.

Trask, B., Allen, S., Massa, H., Wu, M., van den Engh, G. and Fertitta, A. (1994) Studies of metaphase and interphase chromosomes by using fluorescence in situ hybridization. Cold Spring Harbor Symp. Quant. Biol., in press.

van Dekken, H., van Rotterdam, A., Jonker, R., van der Voort, H.T.M., Brakenhoff, G.J. and Bauman, J.G.J. (1990) Confocal microscopy as a tool for the study of the intranuclear topography of chromosomes. J. Microsc. 158, 207–214.

van den Engh, G., Sachs, R. and Trask, B. (1992) Estimating genomic distances from DNA sequence location in cell nuclei by a random walk model. Science 257, 1410–1412.

van Ommen, G.J. (1993) First report of the HUGO YAC committee. Genome Priority reports. New York, pp. 885–888.

Vogelstein, B., Pardoll, D.M. and Coffey, D.S. (1980) Supercoiled loops and eucaryotic DNA replication. Cell 22, 79–85.

Vooijs, M., Yu, L.-C., Tkachuk, D., Pinkel, D., Johnson, D. and Gray, J.W. (1993) Libraries for each human chromosome, constructed from sorter-enriched chromosomes by using linker-adapter PCR. Am. J. Hum. Genet. 52, 586–597.

Weier, H.G., Segraves, R., Pinkel, D. and Gray, J.W. (1990) Synthesis of Y chromosome-specific labeled DNA probes by in vitro DNA amplification. J. Histochem. Cytochem. 38, 421–426.

Wiegant, J., Ried, T., Nederlof, P., van der Ploeg, M., Tanke, H.J. and Raap, A.K. (1991) In situ hybridization with fluoresceinated DNA. Nucleic Acids Res. 19, 3237–3241.

Wiegant, J., Kalle, W., Mullenders, L., Brookes, S., Hoovers, J.M.N., Dauwerse, J.G., van Ommen, G.J.B. and Raap, A.K. (1992) High-resolution in situ hybridization using DNA halo preparations. Hum. Mol. Genet. 1, 587–592.

Wiegant, J., Wiesmeijer, C.C., Hoovers, J.M.N., Schuuring, E., d'Azzo, A., Vrolijk, J., Tanke, H.J. and Raap, A.K. (1993) Multiple and sensitive fluorescence in situ hybridization with rhodamine-, fluorescein-, and coumarin-labeled DNAs. Cytogenet. Cell Genet. 63, 73–76.

Willard, H.F. and Waye, J.S. (1987) Hierarchical order in chromosome-specific human alpha satellite DNA. TIG 3, 192–198.

Zirbel, R.M., Mathieu, U.R., Kurz, A., Cremer, T. and Lichter, P. (1993) Evidence for a nuclear compartement of transcription and splicing located at chromosome domain boundaries. Chrom. Res. 1, 93–106.

Molecular Diagnosis Reviews

In Situ Hybridization—Theory and Practice

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In situ hybridization is a technique to determine and localize target nucleic acids in morphologically preserved tissue sections. Recent advances in methods have greatly increased the sensitivity of the technique, and it is currently possible to detect extremely few copies of any given target sequence with nonisotopic methods. In this teaching review, we integrate theoretical background, technical considerations, and guidelines for usage for this important component of molecular diagnosis.

Key words: *In situ* hybridization.

Molecular pathologists are faced with many choices in selecting the best means to measure a given analyte. The choice goes beyond simple technical considerations of whether it is better to measure DNA or RNA; whether quantitation is required; whether such structural changes as point mutations, deletions, polymorphisms, rearrangements, or translocations must be assessed; and, finally, whether the molecular information must be integrated with additional data concerning the anatomic localization or distribution of the analyte. The purpose of this review is to describe our experience with *in situ* hybridization techniques and to provide guidance to when *in situ* hybridization may prove to be the method of choice.

In situ hybridization is a technique to visualize specific nucleic acid sequences in tissue sections. Currently, *in situ* hybridization is used to diagnose infectious diseases and assess neoplastic disorders [1,2]. Unlike polymerase chain reaction (PCR)-based or filter hybridization techniques, *in situ* hybridization allows visualization of analyte sequences in morphologically preserved tissue sections. The importance of direct visualization can be quite significant. For example, localization of Epstein-Barr virus (EBV) to neoplastic Reed-Sternberg cells in Hodgkin's disease provides an important marker for Reed-Sternberg cells, as well as an increased understanding of the pathogenesis of Hodgkin's disease. Localization to specific subpopulations of cells is not possible with filter hybridization techniques or routine solution-based PCR assays using nucleic acids obtained by tissue disruption as the starting template unless laborious cell-separation procedures are used.

In the past, nonisotopic *in situ* hybridization techniques showed a general lack of sensitivity. Isotopic methods, although more sensitive, required long exposure times, as well as the cumbersome use of radioactivity. Sensitivity has improved remarkably in recent years because of improved probe labeling methods and vastly superior label detection protocols that amplify signal using biotinyl tyramide or DNA polymer-based techniques. In general, *in situ* hybridization detects a threshold of 20 to 50 copies

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of a target sequence per cell [3–6], but sensitivity can be further improved by more sensitive means of detection, such as tyramide-catalyzed signal amplification, discussed later in this review.

Technical Considerations

System Selection

In situ hybridization can be performed using either isotopic or nonisotopic systems. Although radioisotopes offer the highest sensitivity and, particularly with tritium, the highest resolution, they are far less convenient to use than nonisotopic labels. Radioisotopes require special licensing and facilities. Moreover, radioactive probes must be visualized by an autoradiographic process that must be performed in absolute darkness, usually without a safety light. Once coated with autoradiographic emulsion, exposures of up to several weeks can be required. Finally, after processing, the results must be carefully interpreted because of the potential for background radiation or organic chemicals to precipitate background silver grains. In contrast, nonisotopic labels are rapid and convenient, at the expense of some degree of sensitivity and resolution. The relative advantages and disadvantages of each system are summarized in Table 1. Because nonisotopic systems avoid the disadvantages of radiation use, they are generally preferred unless extreme sensitivity or quantitation is required. Further discussion in this review focuses on nonisotopic systems.

Fixation and Sectioning

Either formalin-fixed paraffin-embedded sections or frozen sections can be used. Fixation is an extremely critical step in obtaining good results. Underfixation results in loss of hybridization signal and disrupts tissue morphology as the section goes through the steps of an *in situ* hybridization protocol. Immediate fixation after removal is critical to preserve RNA in a tissue. For optimal fixation, immediately immerse a freshly cut 3- to 4-mm tissue slice in 5 to 10 volumes of 10% neutral buffered formalin for 16 to 24 hours, then embed it in paraffin. Prolonged fixation over 3 to 4 days will unpredictably decrease signal intensity. Paraformaldehyde, 4%, in 0.01 M phosphate-buffered solution, pH 7.4, can also be used as a fixative in place of 10% neutral buffered formalin. The paraformaldehyde solution may be prepared fresh or can be stored at 4°C for about 2 weeks. Prolonged storage will result in the formation of formic acid, resulting in inadequate fixation.

Formaldehyde, which cross-links, is the fixative of choice for *in situ* hybridization [7–9]. Extensive cross-linking produced by other fixatives, such as glutaraldehyde, generally results in decreased hybridization signals. Fixatives containing heavy metals or picric acid, such as Zenker's or Bouin's solution, are also generally not suitable for this technique [10]. We successfully analyzed EBV on B5-fixed bone marrow sections, although the final signal intensity was somewhat weaker than tissues similarly fixed in formalin. Tissues snap frozen in liquid nitrogen and stored at −80°C can be sectioned at 5-μm thickness and fixed in ice-cold acetone or 95% ethanol for 30 minutes before *in situ* hybridization. Frozen sections yield suboptimal morphology and are generally not recommended for *in situ* hybridization.

All sections should be placed on organosilane- or poly-L-lysine–coated glass slides. This ensures

Table 1. System Choice, Isotopic Versus Nonisotopic

	Advantages	Disadvantages
Isotopic, e.g., ^{35}S, ^{3}H, ^{33}P labels	High sensitivity, easy detection, efficiency of probe labeling can be estimated easily by counting, can be used for quantitation by grain counts (^{35}S)	Additional equipment for radioactive use, radiation exposure, variable exposure times, and resolution depending on label, relatively short half-lives of the labels needing repeated labeling and quality control protocols, batch to batch variability in labeling efficiency, disposal issues
Nonisotopic, e.g., biotin digoxigenin, FITC, dinitrophenyl, alkaline phosphatase labels	Good sensitivity, no radioactive exposure, long half lives of the probes, easier quality control and reproducible labeling efficiency, good cellular resolution and morphology, permanent color retention, no disposal issues	Difficulty in detecting extremely low copy sequences, background problems with tissues endogenously rich in the label moiety (biotin), additional detection steps, exact quantitation of target template difficult

adequate adherence of the section and prevents sections from falling off during subsequent processing. For RNA *in situ* hybridization, gloves should be worn while sectioning the blocks, and a new clean microtome blade must be used to reduce potential RNase contamination. RNA is adequately preserved in formalin-fixed and paraffin-embedded tissue sections, but becomes increasingly susceptible to ubiquitous RNases after protease treatment and antigen-retrieval methods. Paraffin-embedded sections are deparaffinized in xylene and hydrated in a graded series of ethanol per usual histological practice.

Target Retrieval

Nucleic acids must be unmasked before formalin-fixed tissue sections can be hybridized with the probe. Unmasking is not generally necessary for frozen sections. Proteinase K, pepsin, trypsin, and pronase E are some of the common proteases used to unmask nucleic acids. Protease digestion is a critical step; under- or overdigestion can greatly affect both signal intensity and morphology. Generally, a good starting procedure is to digest deparaffinized sections for 15 to 30 minutes at 37°C in 10 μg/mL proteinase K in 50 mM TrisHCl, pH 7.5. The digestion time depends on tissue type and whether a slide warmer or water bath is used. The concentration of enzyme and duration of digestion should be adjusted empirically depending on tissue type and duration of fixation. Longer fixation times in formalin require longer protease digestions. If a slide warmer is used, it is preferable to reduce the digestion duration. Temperature control is an important consideration. Care must be taken not to reduce the digestion temperature inadvertently; otherwise, increased background and unacceptable variation within and among specimens might result. For digestion in a water bath, fill a Coplin jar with buffer warmed to 37°C in a water bath and add the required amount of enzyme (e.g., a proteinase K stock at 20 mg/mL), then plunge the slides into the Coplin jar. Note that introduction of the slides will reduce the temperature of the solution; therefore, insert a thermometer into the digestion solution and time the duration of digestion only after the solution reaches 37°C. Digestion is generally stopped by washing the slides extensively in a buffer, such as Tris-buffered saline (100 mM TrisHCl, pH 7.5; 150 mM NaCl). For pepsin digestion, a good starting procedure is to digest the sections in 0.1% (w/v) pepsin in 0.01 N HCl (pH ~2.0) at 37°C for 15 to 30 minutes. Pepsin digestion can simply be stopped by washing the slides in Tris-buffered saline (TBS) or phosphate-buffered saline (PBS) at pH 7.5. Enzymatic unmasking is sufficient for routine applications, but is generally not adequate if extremely high sensitivity is required, such as with single-copy detection of human papilloma virus (HPV) 16. In these situations, heat-induced retrieval is necessary, akin to antigen retrieval in immunohistochemistry. For HPV analysis, we steam deparaffinized sections by the capillary gap method in 0.01 M citrate buffer, pH 6.0, at 95°C for 20 minutes and let the sections cool to room temperature in citrate buffer for an additional 20 minutes. This method opposes two slides, leaving a gap of approximately 70 to 100 μm between them. Vertical immersion of the two slides permits the citrate buffer to rise through capillary action and bathe the sections. Steaming by the capillary gap method is a more uniform and controllable method of target retrieval in comparison to microwave retrieval methods. After heat-induced unmasking, the sections are treated with 5 μg/mL proteinase K at room temperature in 50 mM TrisHCl, pH 7.5, for 5 minutes and then washed extensively in buffer. Although the exact mechanism by which heat treatment works is unknown, the heat is likely to denature proteins, increase exposure of potential protease cleavage sites, and, after the ensuing protease digestion, render the nucleic acids more accessible to the labeled probes.

In situ Hybridization Probes

Whether isotopic or nonisotopic, all *in situ* hybridization analyses use one of three distinct types of probes. Each probe type possesses distinct advantages and disadvantages, which requires careful consideration of the analytic goals to match the intended use with the most appropriate probe type. For example, oligonucleotide probes are often the most specific and may be the only means of differentiating single base changes. The tradeoff, however, is that at the same time, oligonucleotide probes are the least sensitive because their short, defined length limits the absolute amount of label that can be incorporated, and the short length also diminishes the stability of the hybridized probe-target complex. Similarly, double-stranded DNA

probes are more easily prepared and less prone to degradation, but at the same time may be less sensitive because of re-annealing of the probe strands. Moreover, labeling by nick translation or random priming produces a distribution of probe lengths, rather than a single defined length. The properties, advantages, and disadvantages of each probe species are described in Table 2.

In general, DNA or RNA probes routinely used for *in situ* hybridization should be less than 400 bp in length to facilitate tissue penetration. Short oligonucleotides (15 to 40 nucleotides) can also be used as probes, but it is difficult to amplify the signal sufficiently for detection given the relatively low amount of label in any individual oligonucleotide. Oligonucleotides pose additional problems from a kinetic standpoint. Because relatively few bases are engaged in hydrogen bonding to stabilize the hybridized complex, oligonucleotides may more quickly and readily dissociate during processing than would larger probes, spuriously decreasing signal. Both enzymatic and direct chemical coupling reactions can be used to label oligonucleotides. Short oligonucleotides in general are labeled by tailing the 3′ end with a series of bases bearing biotin, digoxigenin, or 2,4-dinitrophenyl. In one study, 2,4-dinitrophenyl–labeled oligonucleotides yielded the strongest signals [11]. Probes should target unique sequence regions in the target DNA or RNA. Such computer programs as Oligo 5.0 (Molecular Biology Insights, Inc., Cascade, CO) are very useful for identifying suitable regions to target in a given sequence and ruling out hybridization to repetitive sequences or other known sequences in such databases as GenBank.

Probe length may influence the quality of the results. Longer probes offer the advantage of increased signal amplification because of a greater number of labeled nucleotides and slower rates of dissociation owing to the larger number of residues engaged in hydrogen bonding. Longer probes offer several methodological approaches to labeling, including nick translation, random priming, and PCR. The first step is to clone the sequence to be used as a probe into one of the several available vectors. TA vectors (Invitrogen, Carlsbad, CA) offer the best choices because of the ease of cloning PCR products. Taq polymerase adds a template-independent A residue at the 3′ ends of both the strands of a PCR product. The TA vectors are supplied linearized in the multiple cloning region, with a T overhang at the 5′ ends of both strands. If the vector and PCR product are mixed in an optimum ratio in the presence of DNA ligase, an efficient ligation can be expected in this system. TA vectors are multifunctional. The insert in the vector can be sequenced using standard M13 forward and reverse primers. Also, TA vectors generally possess

Table 2. Types of Probes

	Advantages	Disadvantages
ds cDNA probes	Stable; easily labeled by random priming, nick translation or by PCR; high specific activity; increased detection sensitivity due to higher number of label moieties; RNase activity not a consideration	Need to subclone sequence into vector requiring molecular biology facilities; can self-anneal reducing hybridization efficiency to target even after an initial denaturation step; only one strand (antisense) is available for hybridization to mRNA; tissue penetration problems if longer probes are used; vector sequences can produce nonspecific hybridization
ss RNA probes	Defined length; single-stranded, high specific activity; easily synthesized and labeled by *in vitro* transcription; greater stability of RNA:RNA hybrids and hence high specificity of hybridization because more stringent washing can be used; being single-stranded (antisense), entire amount of probe is available for hybridization to the target	Molecular biology facilities needed; susceptibility to degradation by ubiquitous RNase contamination; measures to control and minimize RNase activity required; more "sticky" than ds DNA probes and hence increased backgrounds; tissue penetration problems if longer probes are used
Oligonucleotide probes	Defined length; single-stranded, easily synthesized on an automated synthesizer; excellent tissue penetration; great specificity allows differentiation between very closely related but distinct sequences	Prior complete sequence information needed; detection sensitivity lower due to lesser number of label moieties; less stable hybrids due to short length of hybrid, hence very stringent wash conditions decrease final signal intensity

ds cDNA, double-stranded complementary DNA; ssRNA, single-stranded RNA

T7 and Sp6 RNA polymerase promoter sequences on either side of the insert, facilitating single-stranded RNA probe generation. Once the orientation of the insert is determined, these RNA promoters can be used to generate sense and antisense RNA probes with an appropriate *in vitro* transcription system. Recently, TA vectors using attached topoisomerase II to catalyze ligation have become available (Invitrogen). Topoisomerase-activated vectors do not require DNA ligase. In our experience, PCR products with an A overhang ligate very efficiently into such vectors.

Long double-stranded DNA probes are generally labeled with biotin, digoxigenin, or fluorescein isothiocyanate (FITC). The label moiety is covalently linked to dUTP in commercially available labeling mixes. DNA probes can be end-labeled if the 5′ ends of one or both PCR primers used for probe synthesis are labeled, or they can be internally labeled by PCR. Labeling the probe internally is preferred because a greater number of label molecules are incorporated into the probe. A molar ratio of 1:3 of biotin or digoxigenin dUTP to dTTP in the PCR reaction generally gives good labeling.

Labeled single-stranded RNA probes can be generated by *in vitro* transcription. The probe sequence is subcloned into a vector suitable for transcription of the insert from RNA polymerase promoters. Linearized vector is used as a template and 1 μg of the template generates about 10 μg of labeled single-stranded RNA probe by *in vitro* transcription. Several *in vitro* transcription systems are commercially available (Roche Molecular Biochemicals, Indianapolis, IN; Life Technologies, Gaithersburg, MD). The advantage of RNA probes is that they are of defined length.

After probe labeling or *in vitro* transcription reactions, unincorporated labeled nucleotides are removed by ethanol precipitation or column-based methods. Excellent column-based methods are available for probe cleanup (Qiagen, Valencia, CA; Roche Molecular Biochemicals). RNA probes dissolved in RNase-free water and stored at −80°C are stable for at least 2 to 3 years. DNA probes are dissolved in 10 mM TrisCl, pH 8.0, and stored at −20°C. Probes can also be diluted in hybridization solution and stored at −20°C.

Before use, the efficiency of probe labeling needs to be checked. This can be done by spotting a dilution series of the probe alongside known standards on a membrane and using an appropriate detection system. For example, with digoxigenin-labeled probes, one can spot a dilution series of the probe on a nylon membrane, fix the nucleic acid at 80°C for 1 hour, incubate the membrane in anti-digoxigenin-AP conjugate, and visualize the signal colorimetrically with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Roche Molecular Biochemicals). Labeled control standards supplied with the kit are visually compared in intensity with the experimental dilution series.

Hybridization Parameters

Hybridization of the labeled probe to the nucleic acid target in the tissue section must be performed at a reasonably high stringency to ensure specificity. The T_m is defined as the temperature at which half of the probe-target hybrids are dissociated. As with any hybridization, the T_m is affected by salt concentration, temperature, formamide, GC content, and probe length. For DNA probes, hybridization is generally performed at 20°C to 25°C less than the calculated T_m of the hybrid; the T_m will vary with the conditions previously noted, so the hybridization should be performed under the same conditions under which the T_m was estimated or measured. A general formula [12–15] to calculate the T_m for DNA probes greater than 50 nucleotides is the following (note all temperatures in this and following formulas are in degrees celsius:

$$T_m = 81.5 + 16.6\,(\log Na^+) + 0.41\,(\%GC) - 0.61\,(\%\text{ formamide}) - 500/\text{length of probe}$$

For short deoxyoligonucleotides between 14 to 20 bp [13,14,16], the T_d, which is the temperature at which 50% of the short duplexes dissociate when hybridization is performed under standard conditions (0.9 M NaCl), is used to estimate a hybridization temperature. Hybridization is generally performed at 10°C to 15°C less than the calculated T_d.

$$T_d = 4\,(\text{sum of G} + \text{C}) + 2\,(\text{sum of A} + \text{T})$$

For RNA probes hybridizing to RNA templates, the following formula has been used to calculate the T_m [13,14,17]:

$$T_m = 79.8 + 18.5\,(\log NA^+) + 0.58\,(\%GC) + 11.8\,(\%GC)^2 - 0.35\,(\%\text{ formamide}) - 820/\text{length of probe}$$

The stability of the hybrids in decreasing order is RNA:RNA, RNA:DNA, DNA:DNA. Hence RNA:

RNA hybrids would require higher stringency conditions. It is therefore recommended that hybridization be performed at a temperature 15°C to 20°C less than the calculated T_m when RNA probes are used for *in situ* hybridization.

For DNA:RNA hybrids, the formula to calculate the T_m is [13,14,18]:

$$T_m = 79.8 + 18.5 (\log Na^+) + 0.58 (\%GC) + 11.8 (\%GC)^2 - 0.5 (\% \text{ formamide}) - 820/\text{length of probe}$$

As with DNA:DNA hybrids, hybridization is generally performed at 20°C to 25°C less than the T_m.

The T_m of a double-stranded DNA duplex decreases by 1°C to 1.5°C with every 1% decrease in homology for duplexes longer than 100 bp [13,14]. For hybrids shorter than 20 bp, the T_m decreases by approximately 5°C for every mismatched base pair [13,16].

The stability of duplexes is affected by such organic solvents as formamide. Every 1% increase in the formamide concentration reduces the T_m by 0.6°C for a DNA:DNA hybrid. Formamide not only reduces the hybridization temperature, but also tends to reduce background of *in situ* hybridization protocols with RNA probes. Formamide is an organic solvent in which DNA or RNA can be denatured or renatured [19]. Such macromolecules as nucleic acids exist in a helical conformation that has a high degree of intramolecular hydrogen bonding to maintain a formal secondary structure [20]. Such organic solvents as formamide and dimethylsulfoxide (DMSO) cause loss of intra- and intermolecular hydrogen bonding with consequent denaturation and disruption of secondary structure [21]. The net effect is that organic solvents can be used to perform reactions at lower and more convenient temperatures.

Increasing salt concentration or decreasing temperature favors hybridization, whereas decreasing salt concentration or increasing temperature destabilizes the duplexes. As with any hybridization reaction, the repulsive forces of the negatively charged phosphodiester backbones of nucleic acids are progressively exposed as the salt concentration with its potential for charge neutralization is lowered; at high stringency, only the stabilizing forces of a perfect or near-perfect probe match can counteract these repulsive forces.

Dextran sulfate (5% to 10%) is frequently added to the hybridization solution. This results in an increase in the relative concentration of the probe because of the water-binding properties of dextran sulfate and thereby enhances the rate of hybridization. Dextran sulfate also increases the viscosity of the hybridization solution, thereby decreasing the rate at which probe can diffuse away from its target sequences. Denatured salmon sperm DNA saturates sites in which labeled probe could bind nonspecifically and is thus used in hybridization solutions as a blocking agent. Another blocking solution is Denhardt's solution, which at 50× concentration consists of 5 g Ficoll type 400, 5 g polyvinylpyrrolidone, and 5 g bovine serum albumin, Pentex fraction V [14]. For RNA *in situ* hybridization, it is critical that the bovine albumin in Denhardt's solution be free of RNase.

An empirical starting hybridization solution would contain 30% to 50% formamide, 4× sodium chloride/sodium citrate buffer (SSC), 0.1% sodium dodecyl sulfate (SDS), 5% dextran sulfate, 2× Denhardt's solution, and 400 μg/mL denatured salmon sperm DNA. Probe concentrations in the hybridization mix generally range from 1 to 10 ng/μL for long probes and 0.5 to 2 μg/μL or 2 to 10 pmol/μL for short oligonucleotide probes. Hybridization times range from about 2 hours to 16 to 18 hours. When optimizing a protocol, it is preferable to hybridize for 16 to 18 hours and then decrease the time as necessary; otherwise, it is possible to miss an unoptimized positive result. Hybridization rate is not a factor when overnight incubations are used to allow the reaction to approach equilibrium. Precise timing is difficult to achieve in laboratories processing many slides. It is therefore advantageous to allow probes to approach equilibrium through the use of long hybridization times when performing *in situ* analyses. The rate of hybridization is principally influenced by probe length, which affects both the rate of hybridization and the melting temperature, and probe concentration, with more time required at lower probe concentrations.

Probes are generally added in about 10 to 20 μL hybridization solution per section. For double-stranded DNA probes, after the hybridization solution is applied and the section is coverslipped without air bubbles, the slide should be denatured at 95°C for 5 minutes before incubation at the hybridization temperature. Evaporation is not a problem in coverslipped slides during the short duration of the incubation. For RNA probes, it is sufficient to denature at 65°C to 75°C to release the sec-

ondary structures in the target RNA or the probe and then place the slides at the hybridization temperature. This denaturation step is best performed on a slide warmer with a surface temperature that can be accurately adjusted with a surface thermometer.

There are several important considerations in selecting hybridization conditions. Prehybridization using hybridization solution without probe is generally not necessary in the absence of persistent high backgrounds, in which prehybridization tends to reduce background. RNA probes are generally more "sticky" than DNA probes; immersing the sections in freshly prepared 0.1 M triethanolamine buffer, pH 8.0, containing 0.25% acetic anhydride for 10 minutes at room temperature before hybridization tends to decrease nonspecific probe sticking because the negative charges on the slide are partially neutralized by this solution [22]. Also, tissue backgrounds caused by nonspecific binding of unhybridized RNA probes can be abolished by a brief treatment of the section with RNase A after hybridization. RNase A preferentially cleaves single-stranded RNA in a buffer containing greater than 300 mM Na^+ [22]. Treat the sections with 40 μg/mL RNase A in 2X SSC at 37°C for 15 to 30 minutes and wash extensively in buffer. Hybridization must be performed in a humidified box to maintain the concentrations of all solutions at their proper levels.

Posthybridization Washes

The specificity of the final detection is determined by the posthybridization washes. The degree of stringency needed depends on whether only perfectly matched duplexes should survive the wash or some degree of imperfect matches should be tolerated, such as when probing for closely related gene family members. The stringency of hybridization can be adjusted by posthybridization washing in low-salt buffer at a temperature greater than that of the hybridization temperature. During optimization, a series of salt concentrations and temperatures should be systematically tested using the calculated T_m as a starting point. After optimization, it is preferable to wash the sections in the highest stringency wash and not increase the stringency in stepwise fashion, which will result in somewhat diminished signal. The highest stringency wash should generally be about 8°C to 10°C less than the calculated T_m for the salt concentration of the given stringency wash. In general, 0.2X SSC gives sufficient stringency for longer probes of 50% GC content, depending on the temperature used. For short oligonucleotide probes, 1X SSC might achieve the same results at the identical temperature. After the stringency wash solution has been preheated to the desired temperature, the slides are immersed in it. It is very important to insert a thermometer into the solution and time the duration of wash only after the solution containing the slides reaches the desired temperature. Long probes have a wide window between the probe-target binding T_m and the probe-nonspecific binding T_m. However, for oligonucleotide probes, this window can be very narrow unless PCR is combined with *in situ* hybridization.

Detection

The choice of detection system depends on the label incorporated into the probe and the desired sensitivity. Biotin-peroxidase, antidigoxigenin–alkaline phosphatase, and anti-FITC–alkaline phosphatase systems are used with either biotinylated probes or probes labeled with digoxigenin or FITC. Recently, the catalyzed signal amplification technique [23,24] (Dako GenPoint Kit; Dako Corporation, Carpinteria, CA) has allowed routine increase of the sensitivity of HPV detection by *in situ* hybridization to detect a single integrated copy in SiHa cells. After heat-induced unmasking and a brief proteinase K digestion, sections are hybridized to a proprietary (the length and GC content are not disclosed) type-specific HPV biotinylated probe (Dako Corporation) overnight at 37°C, followed by a stringency wash in 0.2X SSC at 52°C for 15 minutes. The site of hybridization is detected by a catalyzed signal amplification system that consists of sequential application of a primary streptavidin-peroxidase complex, biotinyl tyramide, and a secondary streptavidin-peroxidase complex. The streptavidin of the primary complex binds biotin on the hybridized probe, and the peroxidase of the primary complex oxidizes the tyramide of the biotinyl tyramide complex. Oxidized tryamide reacts with the aromatic groups of certain amino acids in the vicinity of the hybrid, and this results in the deposition of more biotin at the site of hybridization. The secondary streptavidin-peroxidase complex binds additional biotin deposited at the hybridization site. The peroxidase of the secondary complex then generates the brown reaction product with the chromagen, diaminobenzidine

(DAB), in the presence of H_2O_2 as substrate. Thus, brown color indicates the site of HPV sequences in the cells. We found the following conditions to be a useful starting point for detection optimization with the Dako GenPoint Kit, with all steps performed at room temperature in a humidified box:

1. Primary streptavidin: 1:100 dilution, 15 minutes
2. Wash in buffer (TBS-T [Tris-buffered saline containing 0.05% Tween 20]): 3 × 5 minutes
3. Biotinyl tyramide: 15 minutes
4. Wash in buffer (TBS-T): 3 × 5 minutes
5. Secondary streptavidin: 15 minutes
6. Wash in buffer (TBS-T): 3 × 5 minutes

Multiple cycles of detection amplification can be used if necessary, by repeating steps 1 through 4. However, if multiple cycles are used, nonspecific background tends to increase. To minimize this, the primary streptavidin has to be diluted to 1:1,000 to 1:5,000 to the empiric endpoint at which background is reduced while retaining adequate signal. We noticed background levels to be problematic, even with 1:5,000 dilution of the primary streptavidin. The Dako GenPoint kit has a working solution of biotinyl tyramide and secondary streptavidin; hence, optimization at this step has to be performed with primary streptavidin dilutions, if possible. Section drying is a significant cause of increased backgrounds. At no time should the sections be allowed to dry. It is advisable to suction fluids off the slide and immediately add the next reagent. Finally, color development is achieved with DAB. With intense signal amplification, color development occurs within seconds, and the investigator must be prepared to stop the reaction immediately if necessary. It is always advisable to monitor the color development under a microscope. Figure 1A–F illustrates the results of HPV analysis with biotinylated DNA probes (Dako Corporation) followed by a single round of catalyzed signal amplification detection. Note that 1 to 2 copies of HPV16 are detectable in SiHa cells. The biotinyl tyramide–catalyzed signal amplification technique can also be used with digoxigenin probes by using antidigoxigenin conjugated to peroxidase in step 1 of the first cycle in the protocol previously mentioned.

For digoxigenin- or FITC-labeled probes, sections are incubated in a conjugate of antidigoxigenin–alkaline phosphatase or anti-FITC–alkaline phosphatase (Roche Molecular Biochemicals; 1:500 dilution) for 1 hour and washed in TBS-T (Sigma Chemical Co., St. Louis, MO). Subsequently, it is important to saturate the sections with Tris-buffered saline, pH 9.5 (100 mM TrisHCl, pH 9.5; 100 mM NaCl), for 5 minutes to activate the alkaline phosphatase. This step greatly decreases nonspecific background. The color substrate solution (NBT/BCIP; Roche Molecular Biochemicals) is generally prepared in a buffer containing 100 mM TrisHCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$. $MgCl_2$ hastens the color development. Color development is slower than in peroxidase-DAB–based systems, but incubation in the substrate solution can be performed for very long periods of time with minimal increases in background. In our experience, adequate color develops in 2 to 3 hours. The blue precipitate of NBT from Roche Molecular Biochemicals or Novocastra (Novocastra Laboratories, Inc., Newcastle-upon-Tyne, UK) is generally incompatible with organic solvents; hence, slides processed by this method are coverslipped with aqueous mounting media (GlycerGel; Dako Corporation).

Levamisole is added to the color substrate solution to inhibit endogenous intestinal alkaline phosphatase activity. We found detection systems from Roche Molecular Biochemicals (Indianapolis, IN) and Novocastra to be very useful. Figure 2A, B illustrates the results of *in situ* hybridization analysis for EBV and cytomegalovirus (CMV) with FITC-labeled RNA probes followed by detection with anti-FITC–alkaline phosphatase conjugate and NBT/X-phosphate. Figure 2C shows the results of RNA *in situ* hybridization for the pp32-related transcripts in human prostatic adenocarcinoma using digoxigenin-labeled RNA probes and an immunoalkaline phosphatase–based detection system [25].

Washing Buffers

TBS-T is our buffer of choice for washing slides after hybridization and during the detection steps. Slides have to be washed in sufficient buffer with shaking. Adequate washing in TBS-T is very critical to reduce background staining when catalyzed signal amplification is used. Most Tris buffers are available commercially (Sigma Chemical Co.).

Counterstain

We routinely use hematoxylin to counterstain the sections. Counterstaining with hematoxylin greatly aids morphological interpretation in comparison to neutral fast red or methyl green. The brown color of the DAB-peroxidase reaction and the

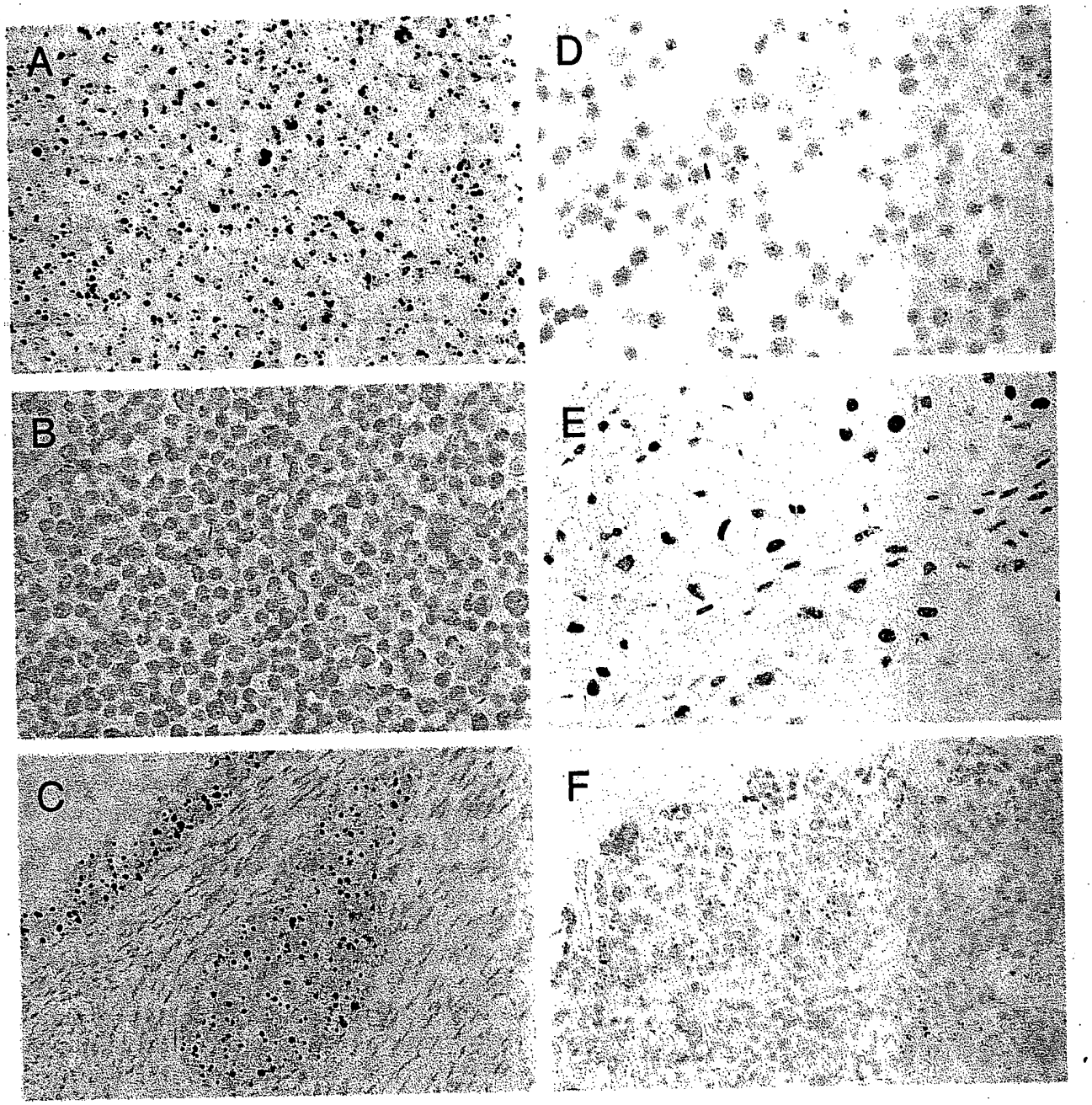

Fig. 1. DNA *in situ* hybridization analysis with biotinylated probes and catalyzed signal amplification detection. (A) Caski cells, HPV 16; (B) HeLa cells, HPV 18; (C) SiHa cells, HPV 16; (D) condyloma, HPV 6/11; (E) squamous carcinoma, cervix, HPV 16; (F) squamous intraepithelial lesion, cervix, HPV 18.

dark blue precipitate of the alkaline phosphatase–NBT/BCIP reaction contrast very well with the light blue color of hematoxylin.

Other Considerations

Distilled deionized water (specific resistance of at least 18 megΩ/cm) is recommended for all steps. For RNA *in situ* hybridization, special precautions have to be taken to minimize RNase contamination. It is preferable to dedicate separate glassware and reagents solely for RNA work. RNase activity can be reduced by treating water with diethylpyrocarbonate (DEPC) (0.1% v/v) followed by autoclaving to remove DEPC. In general, we found distilled deionized water of the type previously mentioned is sufficiently adequate for RNA *in situ* hybridization without further treatment. Unopened

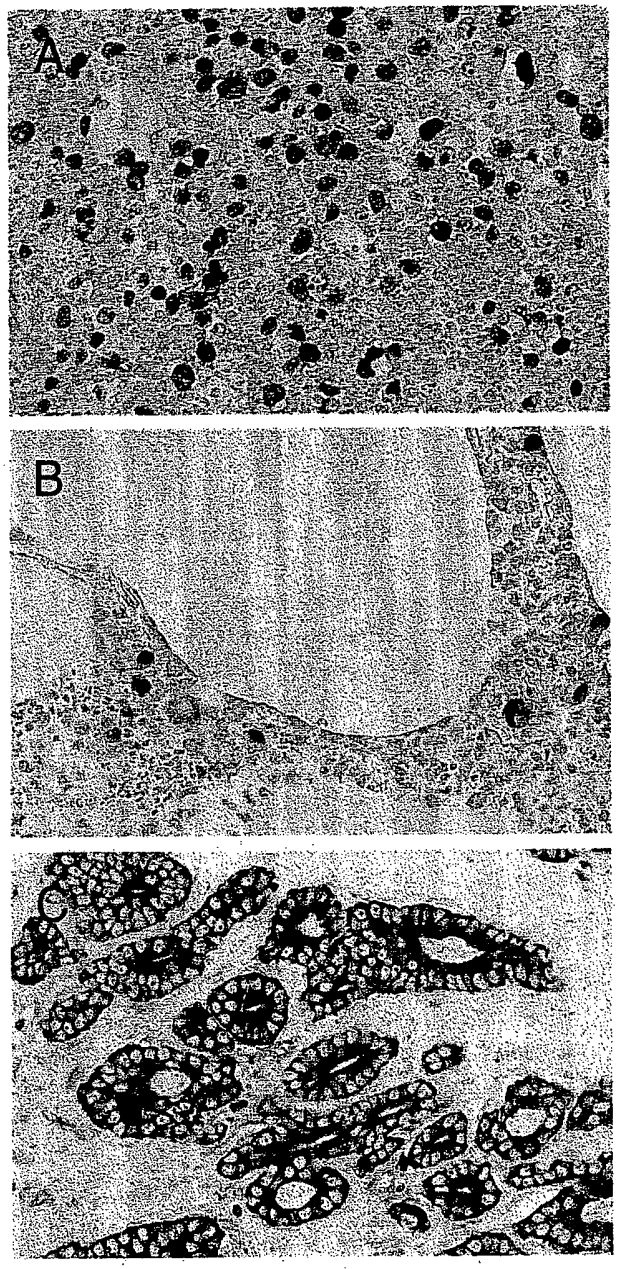

Fig. 2. RNA *in situ* hybridization analysis with FITC- or digoxigenin-labeled riboprobes and immuno-alkaline phosphatase detection. (A) Lymph node, EBV (EBER-1); (B) lung, CMV; (C) prostatic carcinoma, pp32-related transcripts.

sterile plasticware is generally considered RNase free. Gloves should be worn at all times because the major source of RNase contamination is from the hands of the person doing the test.

Controls

Positive and negative controls must be used to evaluate the specificity and significance of the hybridization signal. Preferably, the negative control should be a section known to be negative for the particular target sequence in question. It is preferable to use probes against housekeeping genes or poly-dT probes to check for general preservation of DNA or RNA in tissue sections. Poly-dT probes are more specific for mRNA because they principally associate with the poly A tail. Intact tissue will yield a strong signal, whereas degraded nucleic acids will yield poor and smudged signals. It should be remembered, however, that absence of a signal using these probes does not necessarily preclude detection of a specific target sequence [1]. This may relate to differences in stability of the various sequences determined in part by secondary structure or protein binding. During optimization for *in situ* hybridization with RNA probes, we hybridize serial sections from the same block with the antisense and sense RNA probes. The sense probe can serve as a negative control. However, if regions of the sense probe are complementary to some other RNA molecule, a signal will be obtained. This signal will behave in the same manner as the signal from the antisense probe, and the signal to noise ratio will not increase with increasing stringency washes. In such situations, the overall level of nonspecific hybridization can be assessed by using plasmid probes or probes of random sequence. We always pretreat a serial section with RNase A and then hybridize it with the antisense riboprobe during optimization. If the final signal originates by hybridization of the probe to a target RNA, then it should disappear with this treatment. The labeled probe can also be competed by excess unlabeled probe to check specificity of hybridization. If hybridization is specific, then a decrease in signal intensity should be observed with increasing concentration of unlabeled probe. A serial section that passes through all the steps but with the probe omitted in the hybridization solution is used to assess the level of nonspecific background contributed by the detection system. One can also digest the section with DNase or RNase before hybridization to check for nonspecific binding of the probe to nonnucleic acid structures in the tissue.

Troubleshooting

In general, investigators are faced with two common problems: lack of hybridization signal or ex-

Table 3. Troubleshooting for Lack of Hybridization Signal

Possible Cause	Possible Solution
Inadequate tissue fixation or use of inappropriate fixatives such as glutaraldehyde, Zenker's, or Bouin's solutions	Fix properly, 10% buffered formalin is preferred
Under- or overdigestion with protease	Adjust enzyme concentration, buffer and pH conditions, duration and temperature of digestion; adjust only one parameter at a time
Poorly labeled or degraded probe	Always check integrity of probe and efficiency of probe labeling before use by agarose or acrylamide gel electrophoresis
Inadequate probe concentration	Increase probe concentration or include dextran sulfate in the hybridization solution; use multiple probes along a greater length of the target
Inadequate denaturation of the probe and target before hybridization	Heat sections at 95°C on a slide warmer for 5 minutes for ds DNA probes and 65°C for RNA probes
Hybridization conditions are too stringent	Reduce temperature of hybridization; reduce formamide and increase salt concentration in the hybridization solution
Inadequate duration of hybridization	Try 16 to 18 hours of hybridization before reducing hybridization time
High stringency posthybridization wash	Reduce stringency by increasing salt, decreasing temperature and duration of the wash accordingly
RNase contamination for RNA *in situ* hybridization	Make sure all reagents and labware are RNase free using DEPC treatment for compatible buffers and baking of glassware; sterile plasticware is usually RNase free, but special sets should be reserved
Low copy numbers of the target sequence in the section	Increase sensitivity of the procedure by heat-induced retrieval and/or catalyzed signal amplification detection
Target sequence truly absent in the tissue section	Use appropriate positive and negative control tissues or cell lines for evaluation

cessive background levels. Tables 3 and 4 list some of the possible causes and solutions.

Applications

Applications of *In Situ* Hybridization

In situ hybridization is a powerful technique to identify target sequences in morphologically preserved tissue sections. Currently, the main applications of *in situ* hybridization are analysis of gene expression and detection of infectious agents. Useful examples of gene expression analysis include oncogenes, tumor suppressor genes, growth factors, and cell-cycle regulatory elements. Diagnostically, this technique can be applied to identify mRNA expression in tumors of the endocrine system, especially if the tumor is negative by immunohistochemistry. For example, pituitary adenomas can express prolactin mRNA by *in situ* hybridization, but remain negative by immunohistochemistry [26]. Similarly, gastrin mRNA is readily detected in Zollinger-Ellison syndrome [27], and albumin mRNA has been used to identify tumors of hepatocellular origin [28,29], particularly in indeterminate metastatic lesions. In many cases, *in situ* hybridization analysis supports the results from standard immunohistochemistry, such as in medullary carcinomas of the thyroid [30,31], insulinomas [32], and neuroendocrine tumors [33]. mRNA expression analysis of light chain restriction can complement detection of monoclonality in a lymphoid neoplasm by immunohistochemistry [34]. MUC2 gene expression analysis by *in situ* hybridization has been found to be of prognostic value in tumors of the pancreas and liver [35]. Integrins in ovarian tumors and expression of vascular endothelial growth factor have been recently studied by *in situ* hybridization [36,37]. If rearrangements in the genes occur, resulting in loss of epitopes recognized by an antibody, *in situ* hybridization can still detect regions of the cognate mRNA, depending on the probe target.

In situ hybridization analysis can be used to study a wide range of infections caused by viruses, bacteria, fungi, and parasites. Viral infections that have been a major focus of analysis by this technique include HPV [3–6,38–41], EBV [42–49], CMV [50–54], herpes simplex virus [55,56], hepatitis C virus [57,58], and many others. With HPV analysis, *in situ* hybridization detects not only the virus, but also the type of virus when appropriate

Table 4. Troubleshooting for Excessive Background

Possible Cause	Possible Solution
Inadequate tissue fixation	Fix properly for at least 16 to 24 hours, 10% neutral buffered formalin is preferred
Overdigestion with protease	Adjust enzyme concentration, buffer and pH conditions, duration and temperature of digestion accordingly
Probe still contains unincorporated labeled nucleotides	Purify probe by ethanol precipitation or column methods
Excessive probe concentration	Decrease probe concentration or exclude dextran sulfate in the hybridization solution
Nonspecific binding of RNA probes	Before hybridization, pretreat sections in 0.1 M triethanolamine buffer, pH 8.0, with 0.25% acetic anhydride
Hybridization conditions not sufficiently stringent	Increase temperature of hybridization; increase formamide and decrease salt concentration in the hybridization solution; increase concentration of blocking agents
Low stringency posthybridization wash	Increase stringency by decreasing salt and/or increasing temperature and duration of the wash accordingly
Excessive duration of hybridization	Systematically decrease hybridization duration
Nonspecific probe binding to tissue after hybridization	For RNA probes, treat sections after hybridization with RNase A to eliminate nonspecifically bound single-stranded probe
Inadequate washing between steps	Wash in adequate amount of detergent containing buffer with shaking on a shaker
Excessive amplification at the detection step	Optimize dilution and duration of incubation with the detection reagents
Drying of tissue sections	Avoid by adequately covering the section with the reagents at each step; always incubate in a closed humidified box placed on a level surface
Tissue type, endogenous high levels of peroxidase, biotin, or alkaline phosphatase	Select system accordingly; quench in 3% aqueous H_2O_2 for 20 minutes before hybridization; use blockers such as biotin-block (Dako) or levamisole for alkaline phosphatase systems
Very high copy numbers of the target sequence	Increase posthybridization wash stringency to avoid nonspecific cross-hybridization with other targets
Closely related but not exactly identical target sequences present	Use appropriate labeled oligonucleotide probes instead of longer probes to distinguish the related sequences

probes are used. Because the oncogenic potential of HPV depends on the HPV type, and HPV type-specific vaccines may be available in the near future, identifying the type of HPV can be important. *In situ* hybridization may complement PCR-based assays, especially in HPV testing. A recent study showed that the probability of a sample being positive by either method was not statistically different [41]. In addition, the same study showed that a similar low percentage (around 8%) of cases analyzed were *in situ* hybridization+/PCR− and PCR+/*in situ* hybridization−. At least half the PCR+/*in situ* hybridization− cases were because the type-specific probe was not used in the *in situ* assay. Additionally, *in situ* hybridization provided results for about 8% of the cases that failed to amplify by PCR. Likewise, in some instances, *in situ* hybridization was the only method that could reveal the presence of a virus in neoplastic cells, such as EBV in the Reed-Sternberg cells of Hodgkin's disease [48]. In another setting, EBER-1 mRNA transcripts are expressed to very high (10^5 to 10^6 copies/cell) levels in latent EBV infections. Detection of EBER transcripts by *in situ* hybridization almost approaches the sensitivity of PCR in this setting. Analysis by *in situ* hybridization can also reveal viral presence in cells that do not show classical morphological signs of infection, and especially in specimens from immunocompromised hosts [53,54]. Some other organisms studied by *in situ* hybridization of paraffin-embedded tissues include *Pneumocystis carinii* [59], sexually transmitted disease agents [60], *Helicobacter pylori* [61], legionella [62], leishmania [63], aspergillus [64,65], and mycobacteria [66]. In these instances, analysis by *in situ* hybridization provides rapid results compared with culture methods; in some instances, sequence-based or immunologic identification are the only available modalities for organisms that cannot be readily cultured.

Future Directions

In situ hybridization combines signal detection with anatomic localization in a precise and predictable manner. In comparison to other generally used molecular pathological techniques, only *in situ* hybridization can document where a signal is being produced in a tissue. Eventually, as *in situ* hybridization methods are developed that detect single nucleotide changes, this already powerful technique will increase in versatility and utility. As the Human Genome Project nears completion, all expressed genes in a cell will be known, and it will become necessary to determine exactly which cells in a tissue express a particular transcript. This task can be daunting, given the fact that large numbers of genes are expressed in any given cell. Determination of expression at the protein level by immunohistochemistry first requires the generation of antibodies against the expressed proteins, which is a time-consuming process. Determination of the expression profile by *in situ* hybridization, especially with oligonucleotides, can prove to be a rapid and specific method to achieve this goal in a short period of time. *In situ* hybridization will also be a powerful technique to monitor gene therapy. From a safety and regulatory standpoint, it will become critical to determine the localization, distribution, and expression of therapeutic genes transferred by viral or plasmid vectors into cells or organs, and this can be accomplished with a great degree of specificity and sensitivity by currently available *in situ* hybridization techniques.

Note Added in Proof

Additional formulae to calculate the T_m of nucleic acid hybrids appear in Wetmer JG: DNA probes: Application of the principles of nucleic acid hybridization. Crit Rev Biochem Mol Biol 1991;26:227–259.

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References

1. McNicol AM, Farquharson MA: *In situ* hybridization and its diagnostic applications in pathology. J Pathol 1997;182:250–261
2. Grody WW, Gatti RA, Naeim F: Diagnostic molecular pathology. Mod Pathol 1989;553–568
3. Nuovo GJ: A comparison of slot blot, Southern blot and *in situ* hybridization analysis for human papilloma virus DNA in genital tract lesions. Obstet Gynecol 1989;74:673–677
4. Walboomers JMM, Melchers WJG, Mullnick, et al.: Sensitivity of *in situ* detection with biotinylated probes of human papilloma virus type 16 DNA in frozen tissue sections of squamous cell carcinoma of the cervix. Am J Pathol 1988;131:587–594
5. Nagai N, Nuovo GJ, Friedman D, Crum CP: Detection of papillomavirus nucleic acids in genital precancers with an *in situ* hybridization technique. Int J Gynecol Pathol 1987;6:366–379
6. Shah KV, Howley PM: Papillomaviruses. In Fields BN, Knipe DM, Howley PM (eds): Fields Virology, 3rd ed. Lippincott-Raven, Philadelphia, 1996, pp. 2077–2109
7. McAllister HA, Rock DL: Comparative usefulness of tissue fixatives for *in situ* viral nucleic acid hybridization. J Histochem Cytochem 1985;33:1026–1032
8. Nuovo GJ: Buffered formalin is the superior fixative for the detection of human papillomavirus DNA by *in situ* hybridization analysis. Am J Pathol 1989;134:837–842
9. Tournier I, Bernuau D, Poliard A, Schoevaert D, Feldmann G: Detection of albumin mRNA in rat liver by *in situ* hybridization: Usefulness of paraffin embedding and comparison of various fixation procedures. J Histochem Cytochem 1987;35:453–459
10. Nuovo GJ: Comparison of Bouin's solution and buffered formalin on the detection rate by *in situ* hybridization of human papillomavirus DNA in genital tract lesions. J Histotech 1991;14:13–18
11. Harper SJ, Bailey E, McKeen CM, et al.: A comparative study of digoxigenin, 2,4 dinitrophenyl and alkaline phosphatase as deoxyoligonucleotide labels in non-radioactive *in situ* hybridization. J Clin Pathol 1997;50:686–690
12. Bolton ET, McCarthy BJ: A general method for the isolation of RNA complementary to DNA. Proc Natl Acad Sci USA 1962;48:1390–1397
13. Meinkoth J, Wahl G: Hybridization of nucleic acids immobilized on solid supports. Anal Biochem 1984; 138:267–284
14. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989
15. Bonner TI, Brenner DT, Neufeld BR, Britten RJ: Reduction in the rate of DNA reassociation by sequence divergence. J Mol Biol 1973;81:123–135
16. Wallace RB, Shaffer J, Murphy RF, Bonner J,

Hirose T, Itakura K: Hybridization of synthetic oligonucleotides to $\phi\chi$ 174 DNA: The effect of single-base pair mismatch. Nucleic Acids Res 1979;6: 3543–3656
17. Bodkin DK, Knudson DL: Assessment of sequence relatedness of double-stranded RNA genes by RNA-RNA blot hybridization. J Virol Methods 1985;10:45–52
18. Casey J, Davidson N: Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide. Nucleic Acids Res 1977;4:1539–1552
19. McConaughy BL, Laird CD, McCarthy BJ: Nucleic acid reassociation in formamide. Biochemistry 1969; 8:3289–3295
20. Helmkamp GK, Ts'o POP: The secondary structures of nucleic acids in organic solvents. J Am Chem Soc 1961;83:138–142
21. Marmur J, Ts'o POP: Denaturation of deoxyribonucleic acid by formamide. Biochim Biophys Acta 1961;51:31–36
22. Ausubel FM, Brent R, Kingston RE, et al.: Current protocols in molecular biology. Wiley, New York, 1995
23. Bobrow MN, Harris TD, Shaughnessey KJ, Litt GJ: Catalyzed reporter deposition, a novel method of signal amplification: Applications to immunoassays. J Immunol Methods 1989;125:279–285
24. Komminoth P, Werner M: Target and signal amplification: Approaches to increase the sensitivity of *in situ* hybridization. Histochem Cell Biol 1997;108:325–333
25. Kadkol SS, Brody JR, Epstein JI, Kuhajda FP, Pasternack GR: Novel nuclear phosphoprotein pp32 is highly expressed in intermediate- and high-grade prostate cancer. Prostate 1998;34:231–237
26. McNicol AM, Walker E, Farquharson MA, Teasdale GM: Pituitary macroadenomas associated with hyperprolactinemia. Immunocytochemical and in situ hybridization studies. Clin Endocrinol 1991;35: 239–244
27. Larsson LI, Hougaard DM: Detection of gastrin and its messenger RNA in Zollinger-Ellison tumors by non-radioactive *in situ* hybridization and immunocytochemistry. Histochemistry 1992;97:105–110
28. Yamaguchi K, Nalesnik MA, Carr BI: *In situ* hybridization of albumin messenger RNA in normal liver and liver tumors—identification of hepatocellular origin. Virchows Arch 1993;64:361–365
29. Rajkumar SV, Richardson RL, Goellner JR: Diagnostic value of albumin gene expression in liver tumors: Case report and review of literature. Mayo Clin Proc 1998;73:533–536
30. Neonakis E, Thomas GA, Davis HG, Wheeler MH, Williams ED: Expression of calcitonin and somatostatin peptide and messenger mRNA in medullary thyroid carcinoma. World J Surg 1994;18:588–593
31. Schmid KW, Ensinger C: "Atypical" medullary thyroid carcinoma with little or no calcitonin expression. Virchows Arch 1998;433:209–215
32. Shorrock K, Roberts P, Pringle JH, Lauder I: Demonstration of insulin and glucagon mRNA in routinely fixed and processed pancreatic tissue by *in situ* hybridization. J Pathol 1991;165:105–110
33. Lloyd RV, Jin L: *In situ* hybridization analysis of chromogranin A and B mRNAs in neuroendocrine tumors with digoxigenin-labeled oligonucleotide probe cocktails. Diagn Mol Pathol 1995;4:143–151
34. Inagaki H, Nonaka M, Nagaya S, Tateyama H, Sasaki M, Eimoto T: Monoclonality in gastric lymphoma detected in formalin-fixed paraffin-embedded endoscopic biopsy specimens using immunohistochemistry, *in situ* hybridization and polymerase chain reaction. Diagn Mol Pathol 1995;4:32–38
35. Yonezawa S, Sueyoshi K, Nomoto M, et al.: MUC2 gene expression is found in noninvasive tumors but not in invasive tumors of the pancreas and liver: Its close relationship with prognosis of the patients. Hum Pathol 1997;28:344–352
36. Liapis H, Adler LM, Wick MR, Rader JS: Expression of $\alpha_v\beta_3$ integrin is less frequent in ovarian epithelial tumors of low malignant potential in contrast to ovarian carcinomas. Hum Pathol 1997;28: 443–449
37. Yamamoto S, Yasui W, Kitadai Y, et al.: Expression of vascular endothelial growth factor in human gastric carcinomas. Pathol Int 1998;48:499–506
38. Anderson SM, Brooke PK, van Eyck SL, Noell H, Frable WJ: Distribiution of human papillomavirus types in genital lesions from two temporally distinct populations determined by in situ hybridization. Hum Pathol 1993;24:547–553
39. Lie AK, Skjeldestad FE, Hagen B, Johannessen E, Skarsvag S, Haugen OA: Comparison of light microscopy, *in situ* hybridization and polymerase chain reaction for detection of human papillomavirus in histological tissue of cervical intraepithelial neoplasia. APMIS 1997;105:115–120
40. Autillo-Touati A, Joannes M, d'Ercole C, et al.: HPV typing by in situ hybridization on cervical cytologic smears with ASCUS. Acta Cytol 1998;42:631–638
41. Unger ER, Vernon SD, Lee DR, Miller DL, Reeves WC: Detection of human papillomavirus in archival tissues. Comparison of in situ hybridization and polymerase chain reaction. J Histochem Cytochem 1998;46:535–540
42. Wu TC, Mann RB, Epstein JI, et al.: Abundant expression of EBER-1 small nuclear RNA in nasopharyngeal carcinoma. A morphologically distinctive target for detection of Epstein-Barr virus in formalin-fixed paraffin-embedded carcinoma specimens. Am J Pathol 1991;138:1461–1469

43. Chang KL, Chen YY, Shibata D, Weiss LM: Description of an *in situ* hybridization methodology for detection of Epstein-Barr virus RNA in paraffin-embedded tissues, with a survey of normal and neoplastic tissues. Diagn Mol Pathol 1992;1: 246–255
44. D'Amore F, Johansen P, Houmand A, Weisenberger DD, Mortensen LS: Epstein-Barr virus genome in non-Hodgkin's lymphomas occurring in immunocompetent patients: Highest prevalence in nonlymphoblastic T-cell lymphoma and correlation with a poor prognosis. Danish Lymphoma Study Group. Blood 1996;87:1045–1055
45. Huh J, Seoh JY, Kim SS: Cell block preparation of a Burkitt's lymphoma cell line as a positive control for in situ hybridization for Epstein-Barr virus. Acta Cytol 1998;42:1144–1148
46. Mikata A, Li DX, Kurosu K, Oda K, Yumoto N, Tamaru JI: Reappraisal of the relationship between immunoglobulin heavy chain gene rearrangement and Epstein-Barr virus infection in Reed-Sternberg cells of Hodgkin's disease. Leuk Lymphoma 1997; 28:145–152
47. Shimakage M, Nakamine H, Tamura S, Takenaka T, Yutsudo M, Hakura A: Detection of Epstein-Barr virus transcripts in anaplastic large-cell lymphomas by mRNA in situ hybridization. Hum Pathol 1997; 28:1415–1419
48. Khalidi HS, Lones MA, Zhou Y, Weiss LM, Medeiros LJ: Detection of Epstein-Barr virus in the L & H cells of nodular lymphocyte predominance Hodgkin's disease: Report of a case documented by immunohistochemical, in situ hybridization, and polymerase chain reaction methods. Am J Clin Pathol 1997;108:687–692
49. Li D, Oda K, Mikata A, Yumoto N: Epstein-Barr virus genomes in Hodgkin's disease and non-Hodgkin's lymphomas. Pathol Int 1995;45:735–741
50. Sheehan MM, Coker R, Coleman DV: Detection of cytomegalovirus (CMV) in HIV+ patients: Comparison of cytomorphology, immunocytochemistry and in situ hybridization. Cytopathology 1998;9:29–37
51. Ozono K, Mushiake S, Takeshima T, Nakayama M: Diagnosis of congenital cytomegalovirus infection by examination of placenta: Application of polymerase chain reaction and in situ hybridization. Pediatr Pathol Lab Med 1997;17:249–258
52. Musiani M, Zerbini M, Venturoli S, et al.: Rapid diagnosis of cytomegalovirus encephalitis in patients with AIDS using *in situ* hybridization. J Clin Pathol 1994;47:886–891
53. Nuovo MA, Nuovo GJ, Becker J, Gallery F, Delvenne P, Kane PB: Correlation of viral infection, histology and mortality in immunocompromised patients with pneumonia. Diagn Mol Pathol 1993;2:200–209
54. Walts AE, Marchevsky AM, Morgan M: Pulmonary cytology in lung transplant recipients: Recent trends in laboratory utilization. Diagn Cytopathol 1991;7: 353–358
55. Annunziato P, Lungu O, Gershon A, Silvers DN, LaRussa P, Silverstein SJ: In situ hybridization detection of varicella zoster virus in paraffin-embedded skin biopsy samples. Clin Diagn Virol 1996;7:69–76
56. Wright CA, Haffajee Z, van Iddekinge B, Cooper K: Detection of herpes simplex virus DNA in spontaneous abortions from HIV-positive women using non-isotopic in situ hybridization. J Pathol 1995; 176:399–402
57. Ohsawa M, Tomita Y, Hashimoto M, Kanno H, Aozasa K: Hepatitis C viral genome in a subset of primary hepatic lymphomas. Mod Pathol 1998;11: 471–478
58. Yamada S, Koji T, Nozawa M, Kiyosawa K, Nakane PK: Detection of hepatitis C virus (HCV) RNA in paraffin-embedded tissue sections of human liver of non-A, non-B hepatitis patients by *in situ* hybridization. J Clin Lab Anal 1992;6:40–46
59. Hayashi Y, Watanabe J, Nakata K, Fukayama M, Ikeda H: A novel diagnostic method of *Pneumocystis carinii: In situ* hybridization of ribosomal ribonucleic acid with biotinylated oligonucleotide probes. Lab Invest 1990;63:576–580
60. Horn JE, Quinn T, Hammer M, Palmer L, Falkow S: Use of nucleic acid probes for the detection of sexually transmitted infectious agents. Diagn Microbiol Infect Dis 1986;4:101S–109S
61. Barrett DM, Faigel DO, Metz DC, Montone K, Furth EE: *In situ* hybridization for *Helicobacter pylori* in gastric mucosal biopsy specimens: Quantitative evaluation of test performance in comparison with the CLOtest and thiazine stain. J Clin Lab Anal 1997;11:374–379
62. Fain JS, Bryan RN, Cheng L, Lewin KJ, Porter DD, Grody WW: Rapid diagnosis of legionella infection by a non-isotopic hybridization method. Am J Clin Pathol 1991;95:719–724
63. van Eys GJ, Schoone GJ, Ligthart GS, Laarman JJ, Terpstra WJ: Detection of leishmania parasites by DNA *in situ* hybridization with nonradioactive probes. Parasitol Res 1987;73:199–202
64. Park CS, Kim J, Montone KT: Detection of *Aspergillus* ribosomal RNA using biotinylated oligonucleotide probes. Diagn Mol Pathol 1997;6:255–260
65. Montone KT, Litzky LA: Rapid method for detection of aspergillus 5S ribosomal RNA using a genus-specific oligonucleotide probe. Am J Clin Pathol 1995;103:48–51
66. Arnoldi J, Schluter C, Duchrow M, et al.: Species-specific assessment of *Mycobacterium leprae* in skin biopsies by *in situ* hybridization and polymerase chain reaction. Lab Invest 1992;66:618–623

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Human Genetics

Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries

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Summary. A method of in situ hybridization for visualizing individual human chromosomes from pter to qter, both in metaphase spreads and interphase nuclei, is reported. DNA inserts from a single chromosomal library are labeled with biotin and partially preannealed with a titrated amount of total human genomic DNA prior to hybridization with cellular or chromosomal preparations. The cross-hybridization of repetitive sequences to nontargeted chromosomes can be markedly suppressed under appropriate preannealing conditions. The remaining single-stranded DNA is hybridized to specimens of interest and detected with fluorescent or enzyme-labeled avidin conjugates following post-hybridization washes. DNA inserts from recombinant libraries for chromosomes 1, 4, 7, 8, 13, 14, 18, 20, 21, 22, and X were assessed for their ability to decorate specifically their cognate chromosome; most libraries proved to be highly specific. Quantitative densitometric analyses indicated that the ratio of specific to non-specific hybridization signal under optimal preannealing conditions was at least 8:1. Interphase nuclei showed a cohesive territorial organization of chromosomal domains, and laser-scanning confocal fluorescence microscopy was used to aid the 3-D visualization of these domains. This method should be useful for both karyotypic studies and for the analysis of chromosome topography in interphase cells.

Introduction

Chromosome banding techniques have facilitated the identification of specific human chromosomes and presently provide the major basis upon which chromosomal aberrations are diagnosed. The interpretation of chromosome banding patterns requires skilled personnel and is often technically difficult, especially with respect to detecting minor structural changes and when analyzing complex karyotypes, such as those of highly aneuploid tumor cells (see Cremer et al. 1988b). An additional complexity is that readable metaphase chromosome spreads are sometimes very difficult or impossible to prepare from certain cell types or tissues. Alternative methods for identifying chromosomal aberrations could augment current methods of cytogenetic analysis, particularly if applicable to both mitotic and interphase cell populations.

Over the past few years a considerable body of evidence has been obtained which indicates that the DNA of individual chromosomes occupies focal territories, or spatially cohesive domains, within mammalian interphase nuclei (Cremer et al. 1982; Hens et al. 1983; Schardin et al. 1985; Manuelidis 1985a; Pinkel et al. 1986a). These observations strongly suggest that chromosome-specific probe sets could be used to detect numerical or structural aberrations of chromosomal domains in non-mitotic cells, an approach termed "interphase cytogenetics" (Cremer et al. 1986). Indeed, recent in situ hybridization studies have demonstrated the prenatal diagnosis of trisomy-18 with interphase cells (Cremer et al. 1986) and the detection of numerical chromosomal abnormalities in tumor cell lines (Cremer et al. 1988a) using chromosome-specific repetitive DNAs as probes. Since all chromosome-specific repetitive DNAs reported to date are localized to discrete subregions of each chromosome, this class of DNA probes would be unsuitable for analyses of many types of chromosomal aberrations, e.g., translocations and deletions. However, the ability to detect uniquely the entire spectrum of sequences comprising a specific chromosome would make such analyses possible. Furthermore, such a general labeling technique would allow one to address fundamental questions concerning the spatial organization of chromosomal DNA within interphase nuclei.

In this report we present a hybridization strategy suitable for the specific cyto-staining of individual human chromosomes using commercially available genomic DNA libraries that originated from flow-cytometry sorted human chromosomes (Van Dilla et al. 1986). Suppression of hybridization signals from ubiquitous repeated sequences, such as the *Alu* and *Kpn*I elements, was achieved using total human DNA in a reannealing procedure that is based on rapid reassociation kinetics. Similar principles were used previously to facilitate the selective hybridization of unique sequence subsets from cosmid DNA clones for Southern blotting (Sealey et al. 1985) and in situ hybridization experiments (Landegent et al. 1987). We demonstrate here the specific labeling of individual chromosomes in both metaphase spreads and interphase nuclei, as well as the feasibility of using computer-assisted optical sectioning for 3-D reconstruction of chromosomal domains for the analysis of nuclear topography. In an accompanying paper (Cremer et al. 1988b) we further document the utility of this

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technique to detect chromosomal aberrations in highly aneuploid tumor cells.

Materials and methods

DNA libraries

The following human chromosome genomic libraries were obtained from the American type culture collection: LA01NS01 (chromosome 1), LL04NS01 (chromosome 4), LA07NS01 (chromosome 7), LL08NS02 (chromosome 8), LA13NS03 (chromosome 13), LL14NS01 (chromosome 14), LL18NS01 (chromosome 18), LL20NS01 (chromosome 20), LL21NS02 (chromosome 21), LA22NS03 (chromosome 22), LA0XNL01 (chromosome X). Amplification of these phage libraries on agar plates (using LE 392 cells as the bacterial host), purification of the λ phages and extraction of phage-DNA pools were carried out according to standard protocols (Maniatis et al. 1982).

Preparation of metaphase spreads and fibroblast cells

Phytohemagglutinin-stimulated lymphocytes from a normal adult male (46, XY) were cultured in McCoy's 5A medium (GIBCO), arrested with Colcemid, treated with a hypotonic solution of 0.075 *M* KCl, fixed in acetic acid-methanol and metaphase spreads made by standard procedures. Low-passage normal human foreskin fibroblasts (46, XY) were grown on microscope slides, fixed with paraformaldehyde, and permeabilized as described (Manuelidis 1985b), for study of preparations with a more intact three-dimensional structure.

Preparation of DNAs for in situ hybridization

Insert DNA probes. Genomic DNA fragments from the chromosomal DNA libraries were separated as a pool from the Charon 21A vector arms by digestion with the appropriate restriction enzyme [*Eco*RI (LA libraries) or *Hin*dIII (LL libraries)] followed by preparative electrophoresis in 0.6% agarose gel. The insert fragments were isolated from gel slices by electroelution into an Elutrap (Schleicher and Schuell) and further purified by Elutip-d column chromatography (Schleicher and Schuell). The DNA was then extracted with phenol/chloroform (1:1) and ethanol precipitated. This pool of DNA fragments was labeled either by nick translation using Bio-11-dUTP (Langer et al. 1981; Brigati et al. 1983) or by random primer extension with the multiprime DNA labeling system (Amersham) substituting dTTP with 0.5 m*M* Bio-11-dUTP. Alternatively, the DNA of the chromosome-specific libraries was biotin-labeled directly (without separation of the vector arms) by nick translation.

Probe size. To facilitate probe penetration and to optimize reannealing hybridization, it is desirable to have labeled DNA fragments smaller than 500 nucleotides, with the majority preferentially of 150 to 250 nucleotides in length. DNAse concentrations were empirically established in nick-translation reactions to yield fragments in the desired size range and this was verified by agarose gel electrophoresis. Random primer extensions were also carried out under conditions which yielded a comparable DNA size distribution.

Competitor DNA. Human genomic DNA (from placenta or white blood cells) prepared as described (Davis et al. 1986) as well as salmon testis genomic DNA (Sigma) were digested with DNAse to obtain fragments with the same size distribution as the probe DNA, then extracted with phenol/chloroform and ethanol precipitated. These competitor DNAs were used in varying ratios with probe sequences, as detailed in the Results.

Preannealing and hybridization

Under standard conditions from 5 μg/ml to 30 μg/ml of biotin-labeled DNA, representing library insert fragments, and varying amounts of competitor DNAs were combined, ethanol-precipitated and resuspended in formamide. The probe concentration was adjusted to reflect the relative DNA content of each chromosome target. For example, chromosome 1 contains approximately 5.3 times as much DNA as chromosome 21 (Mendelsohn et al. 1973); thus the probe concentrations used were 30 μg/ml and 5 μg/ml, respectively. When total library DNA was used as the probe mixture instead of purified DNA inserts, 10 times as much labeled DNA was added to compensate for the large amount of vector sequences. In the case of the X-chromosome library, LA0XNL01, only twice as much labeled library DNA was used since the human DNA inserts constitute almost half of the total DNA. For comparative purposes, the concentration of human competitor DNA in the hybridization mixture was varied from 0 to 1.0 mg/ml and salmon testis DNA was added as required to result in a final DNA concentration of 1.0 mg/ml in 50% formamide, 1 × SSC (0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7.0) and 10% dextran sulfate. These solutions were heated at 75°C for 5 min to denature the DNA and then incubated at 37°C for various times to promote partial reannealing. The preannealing step was done in an Eppendorf tube just prior to applying the hybridization mixture to the specimen. Nuclei and chromosome spreads on glass slides were incubated in 70% formamide, 2 × SSC at 70°C for 2 min to denature chromosomal DNA and then dehydrated in a series of ice-cold ethanol (70%, 90% and 100%, each for 3 min). After application of the preannealed probe mixture (2.5 μl/cm^2) to slides prewarmed to 42°C, a coverslip was added and sealed with rubber cement. The samples were then immediately incubated at 37°C in a moist chamber for 10–20 h.

In those cases where paraformaldehyde fixation was used to more optimally preserve the 3-D structure of the specimen (Manuelidis 1985b), the slides were equilibrated in 50% formamide, 1 × SSC (2 × 15 min), excess fluid was removed without permitting the sample to dry, the probe mixture was added (5 μl/cm^2), and a coverslip mounted and sealed with rubber cement. Denaturation of both probe and cellular DNA was done at 75°C for 5 min before hybridization was allowed to proceed overnight at 37°C.

Detection

After hybridization, the slides were washed in 50% formamide, 2 × SSC (3 × 5 min, 42°C) followed by washes in 0.1 × SSC (3 × 5 min, 60°C). Thereafter the slides were incubated with 3% bovine serum albumin (BSA), 4 × SSC for ca. 30 min at 37°C. Detection of the biotinylated probe was achieved using either fluorescein-labeled avidin or avidin-alkaline phosphatase complexes. All detection reagents were made up in 4 × SSC, 0.1% Tween 20, 1% BSA and all washes were car-

ried out in 4 × SSC, 0.1% Tween 20 (3 × 3 min, 42°C). For fluorochrome detection, slides were incubated with 5 μg/ml fluorescein isothiocyanate (FITC)-conjugated avidin DCS (Vector Laboratories) at 37°C for 30 min followed by washes. In rare cases the FITC signal was amplified by incubation with 5 μg/ml biotin-conjugated goat anti-avidin D antibodies (Vector Laboratories) at 37°C for 30 min, followed by washing, a second incubation with 5 μg/ml FITC-conjugated avidin (37°C, 30 min) and a final wash (Pinkel et al. 1986a). For detection by enzyme activity, samples were incubated with 2.5 μg/ml streptavidin, washed, incubated with 2 μg/ml biotin-conjugated alkaline phosphatase (Vector Laboratories), washed again and pre-equilibrated in AP-buffer 9.5 (100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, 50 m*M* $MgCl_2$) for 2 × 5 min at room temperature. The enzyme reaction was carried out in AP buffer 9.5 containing 330 μg/ml of nitroblue tetrazolium (NBT) and 165 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at 37°C for 0.5–1 h and stopped by incubation in 2 × SSC. All preparations were counterstained with 200 ng/ml 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI), 2 × SSC for 5 min at room temperature and mounted in 20 m*M* Tris-HCl, pH 8.0, 90% glycerol containing 2.3% of the DAPCO antifade, 1,4 diazabicyclo-(2.2.2)octane (Johnson et al. 1982).

Microscopy

Preparations were visualized on a Nikon inverted microscope equipped for DAPI and FITC epifluorescence optics as well as for conventional bright field microscopy (visualization of enzyme-mediated color precipitates). Photographs were taken with Kodak Ektachrome 400 (color) or Ilford XP1 400 (black and white) films.

A Nikon Optiphot microscope mounted to a laser scanning confocal imaging system (Bio-Rad Lasersharp MRC-500; White et al. 1987) was used for computer-assisted optical sectioning and three-dimensional reconstruction of fluorescent labeled chromosome domains in nuclei. Stereo pictures of the three-dimensional reconstructed nuclei were taken directly from the video screen or printed on dry silver film (Imaging Systems Division, 3M Company).

Densitometry

A graphics workstation (VAX station II/GPX, Digital Equipment Corporation) with a frame grabber (ITEX FG-101, Imaging Technology) and a Dage-MTI-65 video camera with a Zeiss S-Planar 60 mm lens were used as previously described (Manuelidis and Borden 1988). Images were digitized directly from the negatives and stored on disk. Background was removed and polygonal regions around each chromosome were defined. Threshold density levels were used to outline chromosome regions within the defined polygonal areas. Mean density levels within these outlined chromosome regions, R, were determined by the total signal $\int I(x, y)\,\mathrm{d}R$/area R, where $\int I(x, y)$ is the pixel intensity (0–255) at each point within the region R. The threshold background intensity was subtracted from the mean regional density both for labeled chromosome 7 and for background chromosomes. The signal to noise ratio was calculated as mean chromosome 7 signal/mean background chromosome signal.

Results

Figure 1A shows chromosome 7 library inserts labeled with biotin and hybridized to metaphase spreads from normal human lymphocytes without human competitor DNA. Prominent labeling of the two no. 7 chromosomes is observed; additionally, a distinct band-like pattern of hybridization is seen on most of the other chromosomes, and two E-group chromosomes are especially brightly stained. This general chromosomal banding pattern resembles R-banding, and suggests that a significant portion of the background cross-hybridization signal originates from *Alu* repetitive sequences. Previous studies (Manuelidis and Ward 1984) have shown that *Alu* sequences delineate an R-banding pattern, while Giemsa positive-banding profiles are highlighted by *Kpn*I interspersed repeats. A series of pilot studies were therefore undertaken to establish experimental parameters to eliminate the hybridization signal from such repetitive elements.

The kinetics of nucleic acid reassociation in solution are dependent on the total concentration of nucleic acid (Co, in moles of nucleotides per liter) and the time of renaturation (t, in seconds). When reassociation conditions are standardized for temperature (taking into account the formamide concentration), cation concentration and buffer system, the reassociation kinetics are comparable with respect to Cot values. Under defined conditions, the fast reassociating fraction of mammalian genomes containing the highly repetitive DNA is completely reannealed at Cot values between 1×10^{-1} and 5×10^{-1} whereas the intermediate fraction containing the middle repetitive DNA is completely renatured at a Cot value of 1×10^{2} (Britten and Kohne 1968). Thus at a human DNA concentration of 1.0 mg/ml (corresponding to 3×10^{-3} moles of nucleotide per liter), the fast fraction would be renatured in approximately 10 s, whereas the middle repetitive DNA would need more than 9 h to reach complete reannealing. Since the fast fraction of reassociating DNA contains most or all of the ubiquitous repetitive DNA causing cross-hybridization signals, we decided to use a total DNA concentration of 1.0 mg/ml and allow partial reannealing of the probe mixture prior to application to specimens. The optimal renaturation time was determined empirically (see below). This was important, since our in situ hybridization conditions deviate from the standard conditions under which reassociation kinetics are determined (e.g. hybridization in 50% formamide at 37°C corresponds to 0% formamide at about 70°C; dextran sulfate also increases the reassociation time significantly). Furthermore, it was unclear to what degree the middle repetitive DNA contributed to the non-specific signal and therefore should also be prevented from hybridization by the preannealing procedure.

The stringency for the reannealing and in situ hybridization experiments was determined in 50% formamide at 37°C (adapted from standard in situ hybridization protocols) and 1 × SSC [this cation concentration of 0.165 *M* comes close to the concentration used in the kinetics study of Britten and Kohne (1968)]. Competitor human DNA was added in the reassociation procedure to obtain the desired final high DNA concentration and to maintain a high level of repetition of the DNA sequences that should preanneal. While total human genomic DNA represents all the highly repetitive DNA to be removed by preannealing it also contains sequences of the target chromosome, thus the addition of excessive amounts of human DNA would be expected to diminish the chromosome-specific signal. Therefore, we determined first the optimal

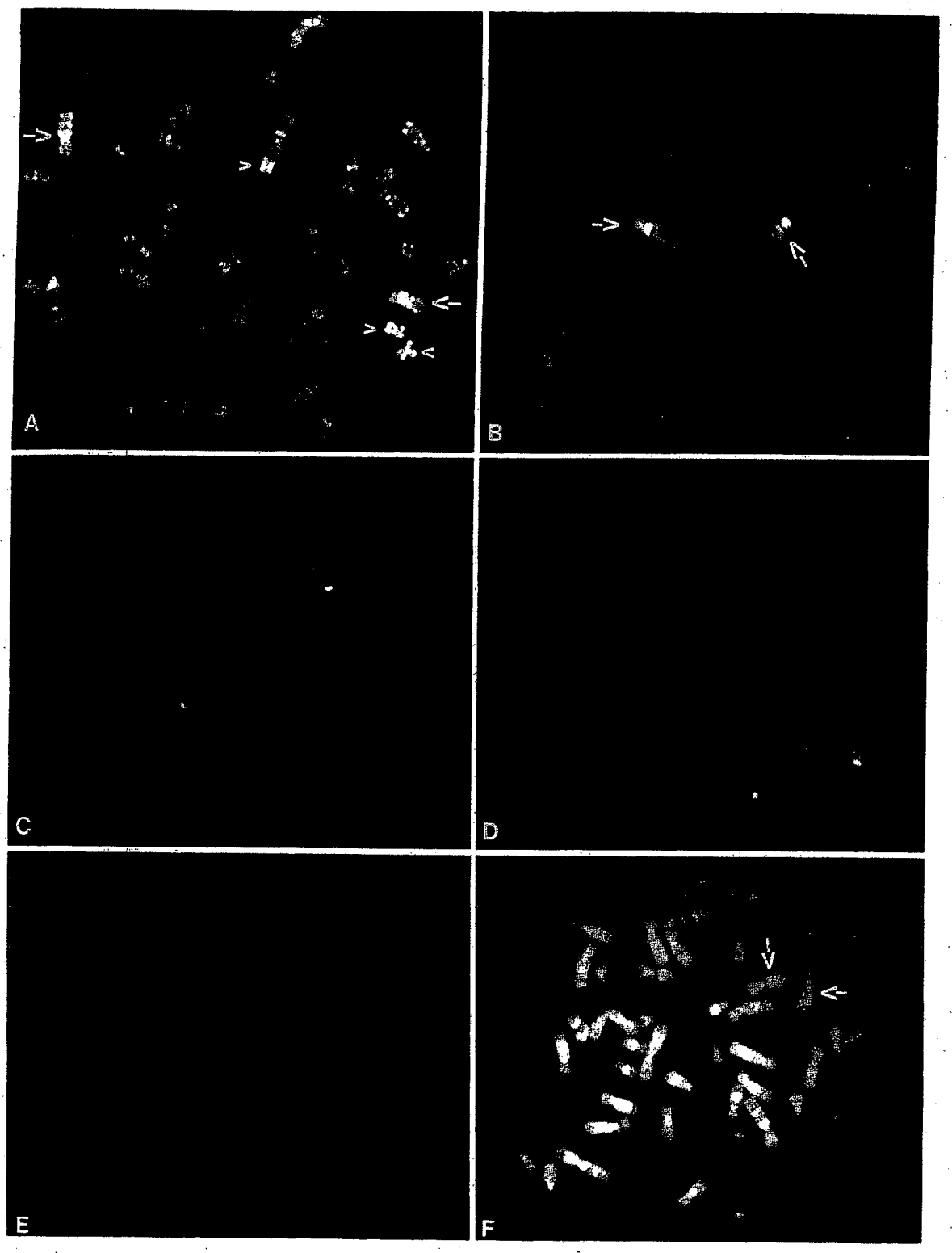

Fig. 1A–F. Suppression of signals from cross-reacting sequences within a chromosome 7-derived DNA library by different concentrations of human competitor DNA. Biotin-labeled chromosome 7 DNA inserts (20 μg/ml) were prehybridized for 20 min with human genomic DNA prior to hybridization with metaphase chromosome spreads and detection with FITC-labeled avidin. Human DNA concentrations were: **A** 0 μg/ml, **B** 50 μg/ml, **C** 100 μg/ml, **D** 200 μg/ml, **E** 1000 μg/ml, **F** same metaphase spread as in E post-stained with DAPI. Genomic salmon DNA was added to each sample to adjust the final DNA concentration to 1.0 mg/ml (see the text for details). The *arrows* mark the target chromosome 7 and the *arrowheads* mark additional strong signals on non-7 chromosomes. All negatives printed were exposed and developed under identical photographic conditions

concentration of total human DNA to use as the competitor. To keep the total DNA concentration constant at 1.0 mg/ml, genomic salmon DNA was added as needed. Salmon DNA shares certain repetitive DNA elements, such as poly dCdA (Hamada et al. 1982) in common with human DNA, but lacks others, most notably the *Alu*- and *Kpn*I repeats. This results in a lower frequency of the latter sequences with increasing amounts of salmon DNA in the reassociation reaction.

Figure 1 shows typical experimental results obtained when 20 µg/ml of the chromosome 7 probe set was denatured together with 50 µg/ml (B), 100 µg/ml (C), 200 µg/ml (D) or 1000 µg/ml (E) of DNAse-digested human genomic DNA which was preannealed for 20 min. Hybridization and detection using avidin-FITC were carried out as described in Materials and methods. From each preparation ten black and white pictures were taken under standardized photographic conditions for densitometric studies (see below). In the absence of human genomic competitor (A) the signal showed little chromosomal specificity. However, with 50 and 100 µg/ml of human competitor DNA an increase of label specificity is readily apparent (Fig. 1B, C). Specific staining of chromosome 7 was achieved with a peak of signal intensity using 100 and 200 µg/ml of human competitor DNA (Fig. 1C, D). Higher concentrations of human DNA caused an apparent decrease of signal intensity, especially at 1000 µg/ml human DNA (Fig. 1E). However, the signal obtained under these latter conditions is still reasonably bright to the observer, but requires a different exposure for optimal illustration (not shown).

A computer-assisted method of quantitative densitometry (see Materials and methods) was used to establish the overall level of labeling specificity. The ratio of fluorescence signal from the target chromosomes of interest to the background fluorescence noise emanating from non-target chromosomes was determined from images digitized from multiple photographic negatives of a DNA titration experiment, as illustrated in Fig. 1. The signal-to-noise ratio obtained with each concentration of human competitor DNA is given in Table 1. Optimal reannealing conditions for suppression of nonspecific signal (using 20 µg/ml of chromosome 7 probe and 100–200 µg/ml human genomic (DNA), gave a signal-to-noise ratio of ca. 8:1. Additional attempts to improve the signal to noise ratio by increasing hybridization stringencies (e.g. 60% formamide or 0.2 × SSC) gave no apparent improvement and led to an overall decrease in signal intensity.

Since about 100–200 µg/ml of human competitor DNA was shown to give the optimal specificity, 200 µg/ml was used for another analysis of signal specificity with respect to the renaturation time (see above). After 0, 2, 5, 10, 20, 40 and 60 min of preannealing, aliquots were taken and used for in situ hybridization experiments as before. As indicated in Fig. 2, specific labeling was obtained for all preannealing times. A small improvement of the signal is seen with increasing renaturation times from 0 to 20 min. Longer renaturation times up to 60 min (not shown) gave no significant improvement in signal strength or chromosome specificity. The subjective impression of a signal improvement with 20 min of preannealing (Fig. 2D) could not be confirmed by a densitometric analysis, carried out as described above, since no significant differences in the signal-to-noise ratio of the different preannealing times were observed (data not shown). We chose 10–20 min as the standard renaturation time in all subsequent experiments. Since a signal is clearly visible at renaturation time 0, the few seconds necessary for transferring the probe mixture to the microscope slide appear to be sufficient to effectively preanneal many of the sequences that cause nonspecific labeling by cross-hybridization. Furthermore, the large excess of single-stranded competitor DNA may efficiently compete with the biotinylated probe sequences for ubiquitous chromosomal target sites during the hybridization reactions. These results demonstrate that the majority of highly repetitive DNA sequences can be sufficiently suppressed to achieve chromosome-specific labeling by in situ hybridization.

Table 1. Densitometric analysis of the suppression of cross hybridization signals by various concentrations of human competitor DNA. Chromosome 7 DNA inserts were labeled with biotin and hybridized in a competitor DNA titration experiment, as illustrated in Fig. 1. *n*, Number of chromosomes from which the mean was determined

Human competitor DNA conc. (µg/ml)	Signal		Noise		Signal-to-noise ratio Confidence interval[c] (99%)
	Pixel[a]	*n*	Pixel[b]	*n*	
0	71.48	8	54.66	26	1.31 ± 0.04
50	74.50	8	37.43	28	1.99 ± 0.07
100	162.64	8	20.06	23	8.11 ± 0.35
200	147.35	8	20.53	26	7.18 ± 0.37
500	89.78	8	18.63	21	4.82 ± 0.28
1000	94.37	8	30.51	17	3.09 ± 0.12

[a] Mean value of pixel intensity of target chromosome
[b] Mean value of pixel intensity of non-target chromosomes (from the same metaphase spreads)
[c] The confidence interval was calculated using Fieller's theorem (Finney 1971)

In certain cases the signal distribution over the entire chromosome shows some variability from experiment to experiment. When the overall signal is decreased some chromosomal subregions show a brighter staining; these signal hotspots generally constitute chromosomal sites that contain known chromosome-specific repetitive sequences. In the experiments shown in Figs. 1 and 2, predominant staining of the centromeric region of chromosome 7 is seen, which corresponds to the chromosome-specific signal of an alphoid repetitive DNA (Waye et al. 1987; see also Cremer et al. 1988b). Apparently, the abundance of these repeated sequences is sufficiently low to prevent their suppression under the conditions used here. The unequal signal distribution can be overcome by amplifying the overall signal using an antibody sandwich technique as described in Materials and methods. Furthermore, when labeling chromosome 1, we frequently saw a predominant staining of the region 1q12 (see Cremer et al. 1988b) that corresponds to the chromosomal site of satellite III DNA (Cooke and Hindley 1979; Gosden et al. 1981). An example of the balanced signal distribution seen after such an amplification step is shown in Fig. 3A.

We tested several commercially available DNA libraries, each representing a single human chromosome, for their ability to specifically label that chromosome under the standardized reannealing conditions described above and with the probe concentrations adjusted for chromosome size (see Materials and methods). Some examples, for chromosomes 1, 4, 7, 13, 18 and 20, as shown in Fig. 3, clearly demonstrate that specific labeling can be achieved with most chromosome libraries. Table 2 lists the libraries tested with their relative scores of labeling specificity. All scores are positive because the chromosome of interest was always decorated. The highest

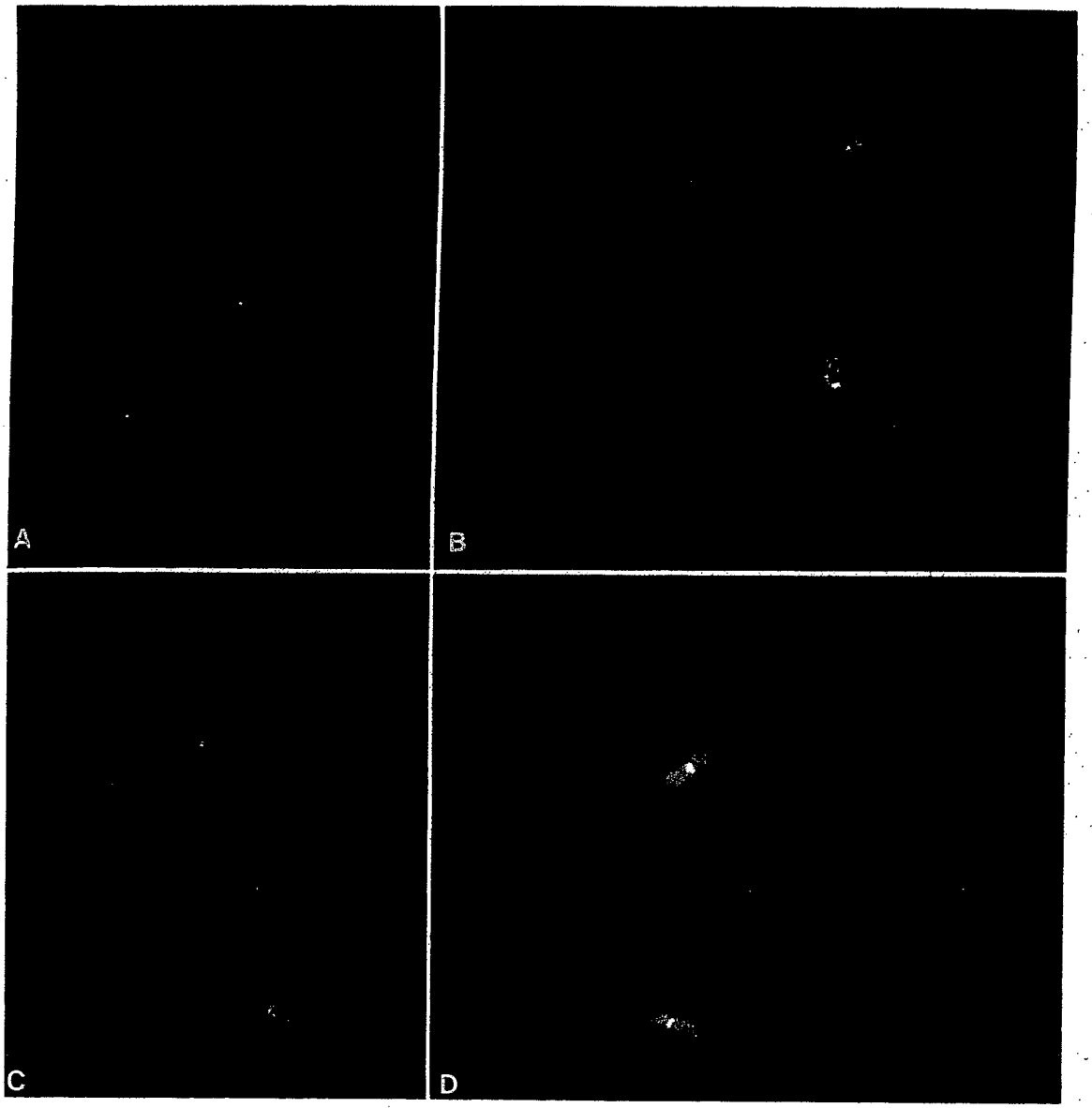

Fig. 2A–D. Effect of preannealing time on the specificity and strength of the hybridization signal. A sample of 20 μg/ml biotin-labeled chromosome 7 DNA inserts was preannealed with 200 μg/ml human competitor DNA for various times: **A** 0 min, **B** 2 min, **C** 5 min, **D** 20 min, before hybridization to metaphase spreads followed by detection with FITC-conjugated avidin

score (4+) is used when no significant cross-hybridization to other chromosomes was observed and the scores decrease (3+ to 1+) with an increasing amount of cross-hybridizing sequences.

All attempts to reduce the additional signals on other chromosomes by varying the experimental conditions failed except in experiments with chromosome 22; in this case higher concentrations of human competitor DNA (700 μg/ml) resulted in a significant improvement of signal specificity. The library exhibiting the lowest chromosome specificity was the chromosome 13 library (Fig. 3E). Multiple minor binding sites on other chromosomes, as well as an exceptionally bright staining of Yq12 were observed; the signal on the Y chromosome was visible using either female or male human DNA as the competitor. None of the experimental parameters tested improved on the overall specificity of this library.

Remarkably, a weak signal or even absence of signal can be observed at the centromeric region of some chromosomes (see chromosomes 4 and 18, Fig. 3C, D). In contrast to chromosomes 1 and 7 which contain chromosome-specific repetitive elements, the centromere regions of chromosomes 4 and 18 apparently contain repetitive sequences, most likely alphoid satellite DNAs, which are very abundant and thus are suppressed by the reannealing technique. However, these chromosomal regions are very small and the effect can only be observed when the corresponding chromosomes are fairly elongated.

We also used biotinylated total library DNA (containing the phage vector sequences) as probes in concentrations adjusted to the amount of human DNA inserts (see Materials and methods). One example is shown in Fig. 3F with the chromosome 20 library. Although good staining of the chromosome of interest generally was achieved, significant nonspecific background on the entire slide was a common problem. Similar results were obtained with plasmid libraries containing human DNA subcloned from the lambda phage

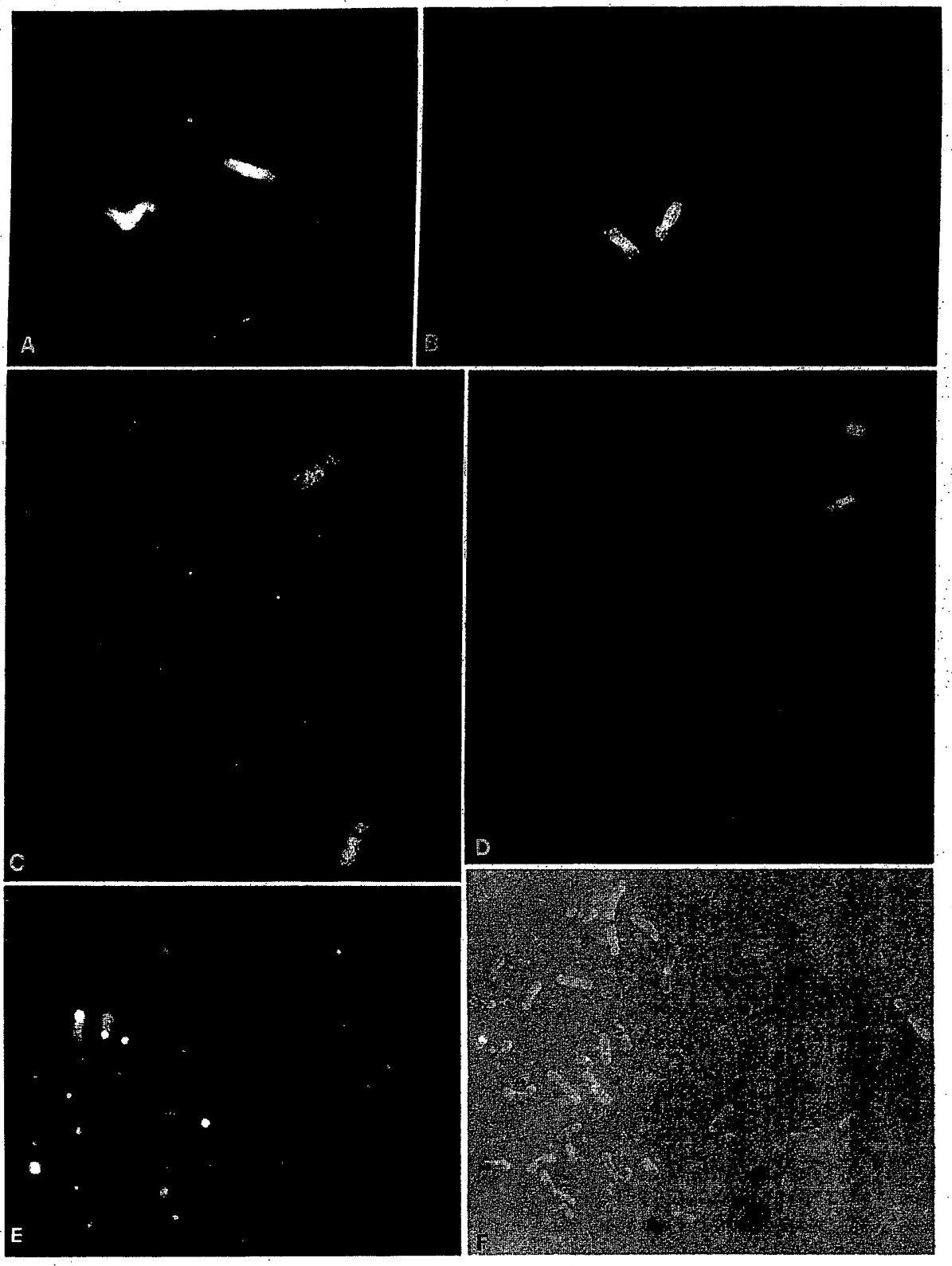

Fig. 3A–F. Decoration of **A** chromosome 1, **B** chromosome 7, **C** chromosome 4, **D** chromosome 18, **E** chromosome 13 and **F** chromosome 20 in normal human lymphocytes. Only the chromosome 13 insert DNA pool shows significant cross-hybridization to other chromosomes after the prehybridization supression step. The detection of chromosome 20 (**F**) was done with the entire chromosome library (including λ phage arms) and detected with avidin-alkaline phosphatase using NBT-BCIP as the enzyme substrate mixture. The signal of chromosome 1 (**A**) was amplified by the sandwich technique of Pinkel et al. (1986a)

libraries (Chieh-ju Tang, P. Lichter, D. C. Ward, unpublished data). In contrast, there was no background problem with the total chromosome library LA0XNL01, which contains a significantly smaller proportion of vector sequences in the probe mixture since the size of the human DNA inserts is much larger.

Table 2. Relative quality of specific chromosome labeling in situ using preannealed biotinylated library DNA inserts

Chromosome	Library used (ATCC designation)	Relative specificity of in situ hybridization signal[a]
1	LA01NS01	3+
4	LL04NS01	4+
7	LA07NS01	4+
8	LL08NS02	4+
13	LA13NS03	1+
14	LL14NS01	2+
18	LL18NS01	4+
20	LL20NS01	4+
21	LL21NS02	3+
22	LA22NS03	3+[b]
X	LA0XNL01	4+

[a] See the text for score definition

[b] Under standard preannealing conditions the chromosome 22 library gave a score of +1; a value of +3 was achieved only with a human competitor DNA concentration of ≥ 700 µg/ml (total DNA concentration 1.0 mg/ml)

The suppression of repetitive sequences by this reannealing technique also permits the use of flow-sorted chromosome libraries to detect chromosomal domains within interphase nuclei. Typical examples of results obtained after hybridization of chromosome 1, chromosome 7 and chromosome 18 probe sets to normal human lymphocytes after acetic acid-methanol fixation are shown in Fig. 4. Discrete focal domains of hybridization signal are seen with all libraries that had scores of 2+ or more (see Table 2). Most nuclei ($n \geq 100$ per estimate) exhibited two domains (60%–70%); however, a significant number showed only a single domain (20%–30%) or no hybridization signal at all (5%–10%). Accordingly, ca. 95% of male nuclei exhibited one and ca. 5% showed no hybridization signal when the X chromosome library DNA was used as probe. Notably, no nuclei with three domains were found with any of the chromosomal probe sets tested. In contrast, all metaphase spreads showed the decoration of both chromosome homologs without exception. This interphase variability may reflect, in part, the close juxtaposition of two individual domains in some cells, or the inability to resolve domains that actually occupy different areas within the nuclear volume but are unresolved when examined by two-dimensional imaging methods (see Fig. 4D; for discussion see also Cremer et al. 1988a). The small number of nuclei exhibiting no hybridization signal may be a reflection of suboptimal hy-

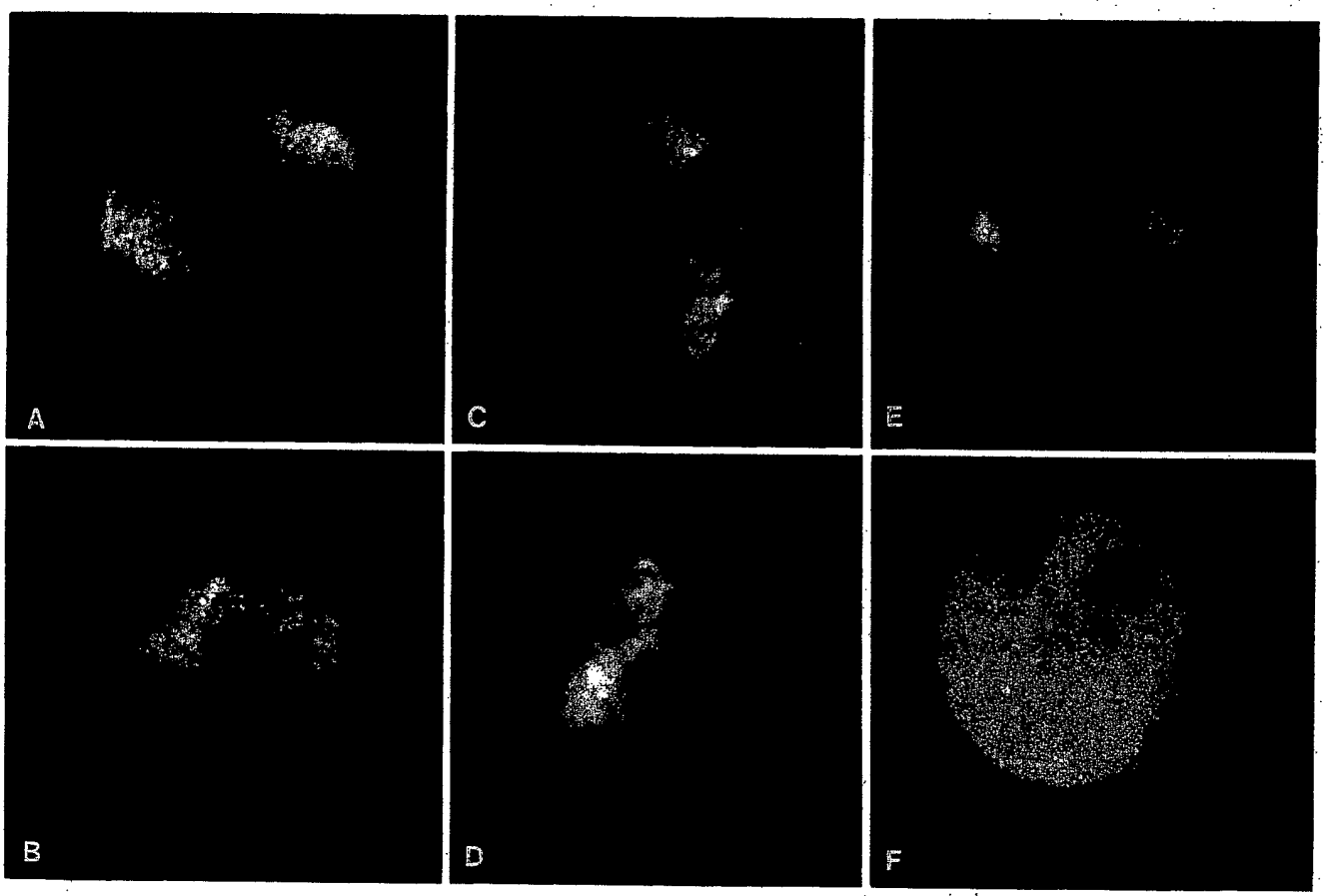

Fig. 4A–F. Chromosome domains in human lymphocyte nuclei delineated by preannealed chromosome library DNA inserts. Hybridization to acetic acid-methanol fixed nuclei was detected by FITC-conjugated avidin (**A–E**) or alkaline phosphatase-conjugated avidin (**F**). Domains are shown for chromosome 1 (**A, B**), chromosome 7 (**C, D**) and chromosome 18 (**E, F**). A predominant staining of the centromere region is seen within the chromosome 7 domains, reflecting preferential hybridization of the chromosome 7-specific alphoid DNA repeat; a similar signal distribution on metaphase chromosomes was also observed in this particular experiment

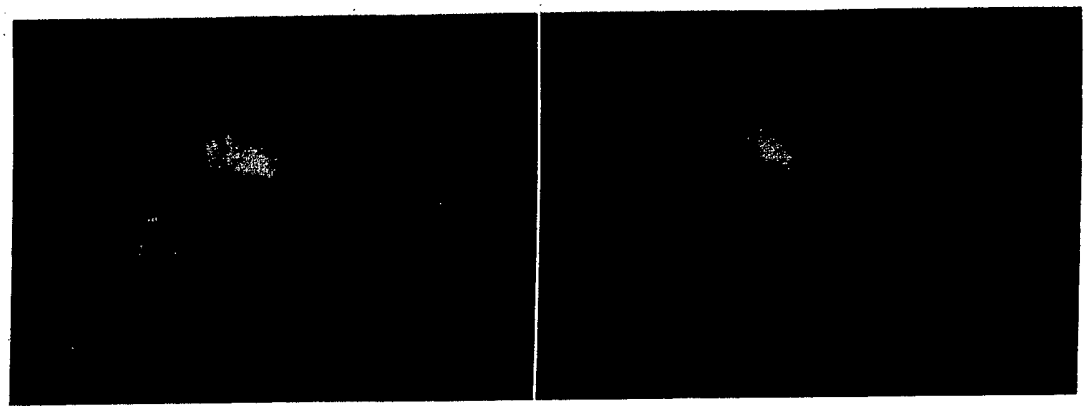

Fig. 5. Stereo picture of a human foreskin fibroblast nucleus highlighting chromosome 7-specific DNA domains. Hybridization to paraformaldehyde-fixed fibroblasts and detection with FITC-labeled avidin was carried out as described in Materials and methods. Using a laser-scanning confocal microscope, 14 continuous optical sections were taken at 0.5-μm intervals and used for 3-D reconstruction. Whereas the lower domain extends in the plain of the print, the upper domain is oriented more upright towards the viewer's eye. A helical substructure of the domains is visible (see the text for discussion). The nucleolus is excluded from the nuclear background staining (here partially framed by the two labeled domains)

bridization conditions. It is of interest to note that the size of the intranuclear domains correlates reasonably well with the relative size of the cognate metaphase chromosome. These observations provide a definitive proof that the DNA of individual chromosomes exhibits a clear territorial organization in the interphase nucleus of a normal human cell.

Acetic acid-methanol fixed nuclear spreads, such as those shown in Fig. 4, clearly retain the territorial organization for each of the chromosomes examined; however, the nuclear structure is not optimally preserved. We have initiated studies with specimens that possess better preservation of 3-D structure using paraformaldehyde fixed human diploid fibroblasts and a laser-scanning confocal fluorescence microscope assembly for 3-D image reconstruction. The cells were fixed and permeabilized as described by Manuelidis (1985b) and hybridized with chromosome library probes as outlined in Materials and methods. The probe-competitor DNA mixture was applied directly to the slide and denatured at the same time as the cellular DNA. Figure 5 shows a stereo pair illustrating the spatial organization of the two chromosome 7 domains within a cell nucleus reconstructed after optical sectioning. Besides the arrangement of the chromosome domains in the nucleus, this figure also shows the frequently observed helical structure of labeled chromatin within chromosome domains. The degree to which this helicity reflects true domain substructure or an artifact reflecting preparation and fixation procedures is currently being investigated. Nevertheless, this preliminary observations establishes the feasibility of using chromosome-specific probes to analyze the topography of chromosomal domains in the interphase cells.

Discussion

In this study we present a method for the efficient and specific labeling of entire individual human chromosomes by in situ hybridization with DNA probes originating from flow-sorted chromosome libraries. Suppression of the signal from the fast reassociating fraction of human genomic DNA was shown, in general, to be sufficient for labeling specificity. Thus the chromosome-specific signal is apparently generated by a combination of middle repetitive, low copy number and unique sequences. A highly specific decoration of the chromosome of interest was achieved with most of the DNA libraries tested, the best of which were the libraries for chromosomes 4, 7, 8, 18, 20 and X. Total recombinant library DNA containing vector sequences can be used as well as purified DNA inserts, although with somewhat higher background noise, thus circumventing the time and labor needed to isolate and purify pools of insert DNA for use as probes. These commercially available DNA libraries are therefore perfectly adequate for chromosomal analysis of the type reported in this and the following paper (Cremer et al. 1988b). Similar results have been observed by J. Gray, D. Pinkel and associates (personal communication) using chromosome 4 and chromosome 21 DNA probe sets. We propose to refer to this method as "chromosomal *in situ* suppression (CISS)" hybridization.

CISS hybridization may also provide a relatively simple screening method to assess the chromosomal specificity of such libraries and to monitor for gross contamination in flow-sorted chromosome fractions. It cannot be determined from our present data whether the observed nonspecific signals are caused by sequences derived from other chromosomes or chromosomal fragments that contaminated the sorted human chromosome fractions, or if they are caused by naturally occurring sequence similarities. However, at least some of the cross-hybridization signals on other chromosomes are apparently produced by sequences that are located on several human chromosomes. For example, a sequence of the satellite III DNA that hybridizes predominantly to chromosome 1 (1q12) also shares homology with several other polymorphic centromeric regions that are present in chromosome 9 and 16 (Gosden et al. 1981). This correlates well with the minor binding sites observed in experiments labeling chromosome 1 (see Fig. 3A). Similarly, part of the cross-hybridization signals of chromosome 22 (not shown) correlates with the hybridization pattern of an α-satellite DNA sequence (McDermid et al. 1986), and the cross-hybridization with probes of chromosomes 13,14,21 and 22 correlates to some extent with the nucleolar organizer regions in the human genome that are known to contain tandemly repeated DNA sequences. In contrast, the expected decoration of the pseudo-autosomal region on the Y chromosome using X chromosome library probes was barely visible.

The fluorescent signals decorating one pair of chromosomes were easily detected and analyzed in mitotic cells, including those usually not accessible to cytogenetic analyses such as early prophase chromosome coils and metaphase spreads where the chromosomes are not well separated from each other (see Cremer et al. 1988b). In addition, interphase domains of the respective chromosomes were distinctly delineated. This constitutes a definite proof for the territorial organization of chromosomes in normal human interphase cell nuclei and extends previous results obtained with rodent-human hybrid lines (Schardin et al. 1985; Manuelidis 1985a; Pinkel et al. 1986a). This territorial organization facilitates the detection of numerical and structural chromosome aberrations in interphase nuclei (see Cremer et al. 1988b). Furthermore, these observations open up a new approach to investigate interphase chromosome topography. We have demonstrated here the successful staining of individual chromosomes in nuclei of paraformaldehyde-fixed fibroblast cells and a three-dimensional imaging of these respective domains. This procedure should enable one to address such general questions as exclusive territorial occupation, specific localization and specific orientation of chromosome domains in different cell types. We are currently investigating these topographic features and their dependence on the cell cycle and stage of differentiation.

Two technical points that are critical for detailed topological studies should be noted. First, it will be essential to perform optical sectioning routinely in order to spatially resolve juxtaposed or overlapping chromosome domains. This can be done using Normarski or epifluorescence optics, image digitizers and computer-assisted 3-D image reconstruction software as previously reported (Agard and Sedat 1983; Pinkel et al. 1986b; Manuelidis and Borden 1988). As shown here, one also can apply laser-scanning confocal microscopy in combination with computer-controlled image reconstruction to define 3-D structure. Confocal microscopy offers the singular advantage that the contribution of out-of-focus signal can be markedly reduced (Cremer and Cremer 1978; Brakenhoff et al. 1979), thus significantly reducing the mathematical computation required for 3-D image reconstruction. The second important requirement is the availabiltiy of multiple labeling techniques for probe detection. Simultaneous visualization of several chromosome domains or subdomains within the same nucleus would allow a much more extensive investigation of nuclear topography. For example, the combination of complete chromosome domain probes with specific subregional probes would facilitate the analyses of both domain orientation and intradomain fine structure. These subregions could be decorated using chromosome-specific repetitive DNA probes, directed to centromeric and telomeric regions (Manuelidis 1984; Rappold et al. 1984; Manuelidis and Borden 1988; Emmerich et al. 1988; Trask et al. 1988) or cosmid clones containing human DNA of known chromosomal map coordinates (Landegent et al. 1987). The application of double labeling techniques to visualize simultaneously entire chromosome domains and centromeric subregions have already proven valuable for detecting chromosomal aberrations in tumor cells, both in metaphase and interphase cells, as outlined by Cremer et al. (1988b).

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References

Agard DA, Sedat JW (1983) Three-dimensional architecture of a polytene nucleus. Nature 302:676–681

Brakenhoff GJ, Blom P, Barends P (1979) Confocal scanning light microscopy with high aperture immersion lenses. J Microsc 117:219–232

Brigati DJ, Myerson D, Leary JJ, Spalholz B, Travis SZ, Fong CKY, Hsiung GD, Ward DC (1983) Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. Virology 126:32–50

Britten RJ, Kohne DE (1968) Repeated sequences in DNA. Science 161:529–540

Cooke HJ, Hindley J (1979) Cloning of human satellite III DNA: different components are on different chromosomes. Nucleic Acids Res 6:3177–3197

Cremer C, Cremer T (1978) Considerations on a laser-scanning-microscope with high resolution and depth of field. Microscopica Acta 81:31–44

Cremer T, Cremer C, Baumann H, Luedtke EK, Sperling K, Teuber V, Zorn C (1982) Rabl's model of the interphase chromosome arrangement tested in Chinese hamster cells by premature chromosome condensation and laser-UV-microbeam experiments. Hum Genet 60:46–56

Cremer T, Landegent J, Brückner A, Scholl HP, Schardin M, Hager HD, Devilee P, Pearson P, Ploeg M van der (1986) Detection of chromosome aberrations in the human interphase nucleus by visualization of specific target DNAs with radioactive and non-radioactive in situ hybridization techniques: diagnosis of trisomy 18 with probe Ll.84. Hum Genet 74:346–352

Cremer T, Tesin D, Hopman AHN, Manuelidis L (1988a) Rapid interphase and metaphase assessment of specific chromosomal changes in neuroectodermal tumor cells by in situ hybridization with chemically modified DNA probes. Exp Cell Res 176:199–220

Cremer T, Lichter P, Borden J, Ward DC, Manuelidis L (1988b) Detection of chromosome aberrations in metaphase and interphase tumor cells by in situ hybridization using chromosome-specific library probes. Hum Genet 80:235–246

Davis LG, Dibner MD, Battey JF (1986) Basic methods in molecular biology. Elsevier, New York Amsterdam

Emmerich P, Loos P, Jauch A, Hopman AHN, Wiegant J, Higgins M, White BN, Ploeg M van der, Cremer C, Cremer T (1988) Double in situ hybridization in combination with digitized image analysis: a new approach to study interphase chromosome topography. Exp Cell Res (in press)

Finney DJ (1971) Statistical methods in biological assay, 2nd edn. Hafner Press, New York

Gosden JR, Lawrie SS, Cooke HJ (1981) A cloned repeated DNA sequence in human chromosome heteromorphisms. Cytogenet Cell Genet 29:32–39

Hamada H, Petrino MG, Kakunaga T (1982) A novel repeated element with Z-DNA-forming potential is widely found in evolutionary diverse eukaryotic genomes. Proc Natl Acad Sci USA 79:6465–6469

Hens L, Baumann H, Cremer T, Sutter A, Cornelis JJ, Cremer C (1983) Immunocytochemical localization of chromatin regions UV-microirradiated in S-phase or anaphase: evidence for a territorial organization of chromosomes during the cell cycle of Chinese hamster cells. Exp Cell Res 149:257–269

Johnson GD, Davidson RS, McNamee KC, Russell G, Goodwin D, Holborow EJ (1982) Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. J Immunol Methods 55:231–242

Landegent JE, Jansen in de Wal N, Dirks RW, Baas F, Ploeg M van der (1987) Use of whole cosmid cloned genomic sequences for chromosomal localization by non-radioactive in situ hybridization. Hum Genet 77:366–370

Langer PR, Waldrop AA, Ward DC (1981) Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. Proc Natl Acad Sci USA 78:6633–6637

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NJ

Manuelidis L (1984) Different central nervous system cell types display distinct and nonrandom arrangements of satellite DNA sequences. Proc Natl Acad Sci USA 81:3123–3127

Manuelidis L (1985a) Individual interphase chromosome domains revealed by in situ hybridization. Hum Genet 71:288–293

Manuelidis L (1985b) Indications of centromere movement during interphase and differentiation. Ann NY Acad Sci 450:205–221

Manuelidis L, Borden J (1988) Reproducible compartmentalization of individual chromosome domains in human CNS cells revealed by in situ hybridization and three dimensional reconstruction. Chromosoma 96:397–410

Manuelidis L, Ward DC (1984) Chromosomal and nuclear distribution of the *Hin*dIII 1.9-kb human DNA repeat segments. Chromosoma 91:28–38

McDermid HE, Duncan AMV, Higgins MJ, Hamerton JL, Rector E, Brasch KR, White BN (1986) Isolation and characterization of an α-satellite repeated sequence from human chromosome 22. Chromosoma 94:228–234

Mendelsohn ML, Mayall BH, Bogart E, Moore DH II, Perry BH (1973) DNA content and DNA-based centromeric index of the 24 human chromosomes. Science 179:1126–1129

Pinkel D, Straume T, Gray JW (1986a) Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 83:2934–2938

Pinkel D, Gray JW, Trask B, Engh G van den, Fuscoe J, Dekken H van (1986b) Cytogenetic analysis by in situ hybridization with fluorescently labeled nucleic acid probes. Cold Spring Harbor Symp Quant Biol 51:151–157

Rappold GA, Cremer T, Hager HD, Davies KE, Müller CR, Yang T (1984) Sex chromosome positions in human interphase nuclei as studied by in situ hybridization with chromosome specific DNA probes. Hum Genet 67:317–325

Schardin M, Cremer T, Hager HD, Lang M (1985) Specific staining of human chromosomes in Chinese hamster × man hybrid cell lines demonstrates interphase chromosome territories. Hum Genet 71: 281–287

Sealey PG, Whittaker PA, Southern EM (1985) Removal of repeated sequences from hybridization probes. Nucleic Acids Res 13: 1905–1922

Trask B, Engh G van den, Pinkel D, Mullikin J, Waldman F, Dekken H van, Gray J (1988) Fluorescence in situ hybridization to interphase cell nuclei in suspension allows flow cytometric analysis of chromosome content and microscopic analysis of nuclear organization. Hum Genet 78:251–259

Van Dilla MA, Deaven LL, Albright KL, Allen NA, Aubuchon MR, Bartholdi MF, Brown NC, Campbell EW, Carrano AV, Clark LM, Cram LS, Crawford BD, Fuscoe JC, Gray JW, Hildebrand CE, Jackson PJ, Jett JH, Longmire JL, Lozes CR, Luedemann ML, Martin JC, McNinch JS, Meincke LJ, Mendelsohn ML, Meyne J, Moyzis RK, Munk AC, Perlman J, Peters DC, Silva AJ, Trask BJ (1986) Human chromosome-specific DNA libraries: construction and availability. Biotechnology 4:537–552

Waye JS, England SB, Willard HF (1987) Genomic organization of alpha satellite DNA on human chromosome 7: evidence for two distinct alphoid domains on a single chromosome. Mol Cell Biol 7:349–356

White JG, Amos WB, Fordham M (1987) An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. J Cell Biol 105:41–48

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Genetics

Rapid detection of human chromosome 21 aberrations by *in situ* hybridization

(Down syndrome/chromosome translocations/prenatal diagnosis/interphase cytogenetics)

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ABSTRACT **Plasmid clones containing up to 94 kilobases of single-copy DNA from band q22.3 of chromosome 21 and a complete pool of insert DNA from a chromosome 21 recombinant library have been used to rapidly detect numerical and structural aberrations of chromosome 21 by *in situ* hybridization in both metaphase and interphase cells. A trisomic karyotype, diagnostic of Down syndrome, is readily detected in nonmitotic cells because the majority of their nuclei exhibit three discrete foci of hybridization, in contrast to normal diploid cells, which show two foci. Chromosomal translocations involving chromosome 21 sequences were also detected with these probes, and the intranuclear location of 21q22.3 DNA sequences in "normal" human brain neurons was established with the plasmid DNA probe set. These results suggest that chromosome 21-specific probes may have utility in clinical diagnostics, especially by facilitating the direct analysis of interphase cells.**

The smallest human autosome, chromosome 21, has been highly relevant to clinical cytogeneticists because trisomy 21 is the primary cause of Down syndrome (1). The recent mapping of the locus for familial Alzheimer disease (2) and the gene for the amyloid β protein (3, 4) to 21q11.2→21q21 has focused additional attention on chromosome 21. Interestingly, Down syndrome patients and familial Alzheimer patients both develop clinical dementias and have similar brain pathology with plaques rich in the amyloid β protein (5, 6). Cytogenetic studies have indicated that only trisomy of subregion 21q22→21qter is required to elicit the Down syndrome phenotype. There is, however, some disagreement as to which part of this subregion is responsible for the complex pathological effects (reviewed in ref. 7).

The majority of Down syndrome patients (≈95%) show three chromosomes 21, and in about 5% of the cases, the trisomy is caused by a Robertsonian translocation (8). Both forms of trisomy are routinely diagnosed by conventional banding techniques Additionally, a small portion (<<1%) of Down syndrome is caused by reciprocal translocation (8). This is difficult to diagnose because the translocated terminal segment of chromosome 21 can be very small, and it is detectable only by high-resolution banding. Another diagnostic complication is the occurrence of trisomy 21 mosaicism. It has been reported that the incidence of chromosome 21 mosaicism is between 1% and 2%, although the actual frequency may be higher (8).

Recent studies (9–15) have shown that the DNA of each chromosome occupies a discrete focal territory within an interphase nucleus. These observations indicate that chromosomal aberrations can be detected directly in nonmitotic cells by *in situ* hybridization using chromosome-specific probes. Indeed, a successful diagnosis of trisomy 18 has been reported by using amniotic fluid cells hybridized with a repetitive sequence probe that, under high stringency conditions, is specific for the centromeric region of chromosome 18 (16). A similar diagnosis of trisomy 21 also has been reported (17) with probe DNA that was derived from flow-sorted chromosome 21 but was not further defined.

DNA probe sets that specifically label the terminal band 21q22.3 or decorate the entire chromosome 21 are reported here, and the application of these probes to the detection of numerical and structural aberrations of chromosome 21 in both metaphase and interphase cells is described.

MATERIALS AND METHODS

DNA Probes. All plasmids contain inserts of human chromosome 21 that were mapped to 21q22.3 (18–22). All inserts were either known (20, 23–25) or verified by Southern blot analysis to be single-copy DNA; the plasmids other than pS2 are subclones derived from a λ phage library (24) or a cosmid library (25). The plasmids are listed in Table 1 with the Human Gene Mapping Workshop symbols (26) and the approximate insert fragment length.

The human chromosome 21 genomic library LL21NS02 was obtained from the American Type Culture Collection and amplified on agar plates as recommended. Phage DNA was prepared and digested with *Hin*dIII, and the DNA inserts were separated from the vector arms by preparative gel electrophoresis in 0.6% agarose. DNA was isolated from gel slices by electroelution; purified by Elutip-d chromatography (Schleicher & Schuell); extracted with phenol/chloroform, 1:1 (vol/vol); and precipitated with ethanol (14).

Human Cells. Metaphase spreads and interphase nuclei were prepared from (*i*) lymphocyte cultures of normal (46, XY) individuals, (*ii*) lymphocytes of Down syndrome (47,+21) individuals, (*iii*) chorionic villi samples cultured for prenatal diagnosis (*ii* and *iii* were provided by T. Yang-Feng, Yale University Cytogenetics Laboratory), and (*iv*) cultures of TC620, an oligodendroglioma-derived pseudotriploid cell line (28). Standard techniques of colcemid treatment, hypotonic treatment, and methanol/acetic acid fixation were used. Biopsy material from the cortical region of a "normal" human brain (46, XX) was fixed, sectioned, and permeabilized as described (29).

***In Situ* Hybridization.** Various combinations of plasmid DNA, labeled with biotin-11-dUTP by nick-translation (30), were used for hybridization at concentrations ranging from 2 to 15 μg/ml depending on the pool size. For example, 15 μg/ml was used when the probe mixture contained 94 kilobases (kb) of insert DNA; the probe concentration was

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Abbreviations: CISS, chromosomal *in situ* suppression; CV, chorionic villi; DAPI, 4',6-diamidino-2-phenylindole.

Table 1. Plasmids with inserts from chromosome 21

Gene symbol	Plasmid	Insert length, kb	Gene symbol	Plasmid	Insert length, kb
BCEI	pS2 (23)	0.6	*D21S56*	pPW520-10R	4.6*†
D21S3	pPW231F	0.8*†		pPW520-11R	1.8*†
	pPW231G	0.7*†	*D21S57*	pPW523-10B	6.5*
D21S23	pPW244D	1.0		pPW523-1H	7.0*
D21S53	pPW512-6B	3.0*		pPW523-5R	2.2*†
	pPW512-8B	3.8		pPW523-10R	3.8*†
	pPW512-1H	2.9*†		pPW523-19R	2.5*
	pPW512-16P	2.7*	*D21S64*	pPW551-8P	1.9*
	pPW512-18P	1.6*		pPW551-12P	4.2*
	pPW512-4R	4.7	*D21S71*	pPW519-10P	0.8
	pPW512-12R	2.0*		pPW519-11P	3.0
D21S55	pPW518-4H	1.6*		pPW519-1R	6.0*
	pPW518-10P	2.9*		pPW519-8R	2.9*†
	pPW518-5R	5.2*†		pPW519-9R	1.7*
D21S56	pPW520-5B	5.0		pPW519-14R	4.0*†
	pPW520-6B	1.0*		pPW519-22R	1.8*

Preparation of plasmid DNA was according to standard protocols (27). Various probe sets were obtained by pooling plasmids (equal molar amounts), resulting in DNA probe complexities of 94 kb (all plasmids listed), 75 kb (plasmids labeled with an asterisk), or 29 kb (plasmids labeled with a dagger).

decreased in proportion to the sequence complexity of the probe mixture. The size of the probe DNA was adjusted to a length of 150–250 nucleotides empirically by varying the DNase concentration in the nick-translation reaction. The hybridization cocktail also contained 50% formamide, 0.30 M NaCl, 0.03 M sodium citrate (pH 7), 10% (wt/vol) dextran sulfate, and on occasion 0.5 mg of sonicated salmon sperm DNA per ml. Simultaneous denaturation of probe and target DNA was carried out at 75°C for 6 min (metaphase spreads) or 94°C for 11 min (tissue slices). Hybridization reactions were incubated at 37°C overnight.

Delineation of individual chromosomes with DNA probes derived from sorted human chromosomes was done by a method termed chromosomal *in situ* suppression (CISS) hybridization as described (14). Briefly, biotinylated chromosome 21 library DNA inserts (5 μg/ml), DNase-digested human genomic DNA (200 μg/ml), and salmon sperm DNA (800 μg/ml) were combined in the hybridization solution, heat-denatured, and partially prehybridized for 10–30 min at 37°C before application to a separately denatured specimen.

Posthybridization washes, detection of hybridized probe by using either alkaline phosphate-conjugated avidin or fluorescein isothiocyanate-conjugated avidin, and photographic conditions were as described (14). When probe sets containing 29 kb or less of target sequence were used, the fluorescein isothiocyanate detection was generally enhanced by one cycle of signal amplification (31).

All quantitative analyses of interphase signals were carried out by using slides from several independent experiments, with more than 100 nuclei being analyzed per slide. Comparison of signals in normal and trisomic samples was done in a blind-study fashion.

RESULTS

Various combinations of cloned DNA fragments from human chromosome 21, previously localized to the 21q22.3 band, were tested for their ability to specifically label the cognate chromosomal region in lymphocyte metaphase spreads and interphase nuclei after *in situ* hybridization. The maximal amount of unique-sequence DNA in the probe set was ≈94 kb; this probe set resulted in a clearly visible labeling of the terminal region of both chromatids of the chromosome 21 homologs (see Fig. 1*B*). These signals were seen unambiguously and without exception in all metaphase spreads, even in spreads of poor quality or from prophase cells (not shown). In normal interphase cells, the majority (65–75%) of nuclei exhibited two signals (see Fig. 1*C*), 25–30% showed one signal, and less than 5% showed no signal (for discussion of signal distribution in interphase nuclei, see refs. 13-15). Nuclei with three signals were found only rarely (<0.2%) and may reflect incomplete hybridization to a few tetraploid cells in the sample. Similar results were obtained with probe sets containing 29 or 75 kb of DNA. With probe sets containing fewer than 20 kb of insert DNA, there were increased numbers of cells with less than two signals. Thus, these probe sets were deemed unsuitable for diagnostic purposes. However, such probes still yielded specific signals on the majority of chromosomes 21, even with a 6-kb single-copy DNA (see Fig. 1*A*), especially when signal amplification was used.

The usefulness of chromosome library DNA CISS hybridization (14) for detecting chromosome 21 was also evaluated. Chromosome 21 was specifically and entirely decorated in normal lymphocyte metaphase spreads, although some additional minor binding sites were seen at or near the centromeric region of other acrocentric chromosomes, especially chromosome 13 (normal karyotype not shown; see Fig. 1*E*). Suppression with additional DNA including the plasmid L1.26, which detects a repetitive DNA located predominantly at the centromeric region of chromosomes 13 and 21 (32), did not efficiently suppress the minor non-21 chromosomal signals. Quantitative evaluation of interphase nuclei signals again showed a negligible portion of nuclei with three signals; however, a significant increase in nuclei with less than two signals was observed (50–60% with two signals, 35–45% with one signal, and 5–10% without a signal). The numerical differences observed with the two different probes can be explained in part by the number of nuclei (up to one of three) that were excluded from the latter analysis because they exhibited larger and more diffuse signals, most likely from more than one chromosome that could not be resolved unambiguously as two separate chromosome domains in a two-dimensional representation. The minor cross-hybridizing sites noted above presented a second experimental complication but did not adversely influence data interpretation.

The optimal (94 kb) plasmid pool as well as CISS hybridization with chromosome 21 library inserts were tested further by using cells containing chromosome 21 aberrations. Both probe sets permitted a fast and unambiguous diagnosis of trisomy 21 in all metaphase spreads from Down syndrome lymphocyte cultures (see examples in Fig. 1 *D* and *E*). Furthermore, the quantitative distribution of hybridization signals in interphase nuclei of the same preparation, analyzed as described above, was similar with either type of probe [<5% of cells with no signal, 5–15% with one signal, 25–35% with two signals, and 55–65% with three signals (Fig. 1 *F–J*)]. Although the library DNA inserts gave up to 15% of four-signal nuclei (compare Fig. 1 *F* and *G*), most likely because of the minor binding sites on other chromosomes, the plasmid pool revealed only a negligible percentage of nuclei (<0.2%) with four signals. These results indicate that trisomy 21 can be detected in a diagnostically meaningful way with small populations of nonmitotic cells.

Embryonic chorionic villi (CV) cells were also investigated with the 94-kb plasmid probe set in a case where the father had a reciprocal t(4;21) translocation. Hybridization to metaphase spreads of the CV cells showed that the translocated chromosome (4pter→4q33::21q11.2→21qter) was indeed inherited by the fetus (see Fig. 1 *L* and *M*). The signals in the interphase cell nuclei (see Fig. 1*K*) of the CV cells had a distribution that paralleled that of cells with a normal karyotype (see above), indicating a balanced representation of

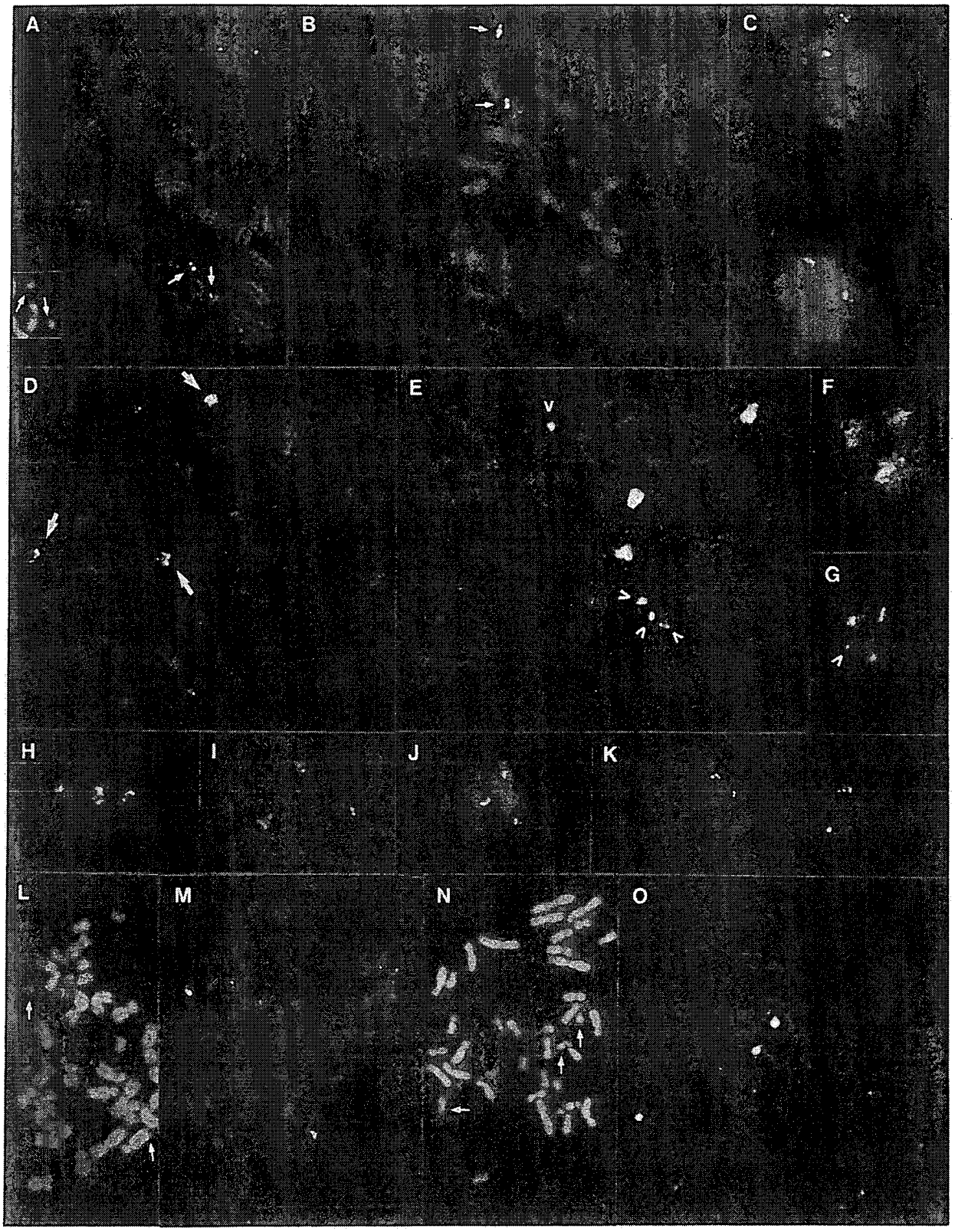

FIG. 1. Specific labeling of human chromosome 21 by *in situ* hybridization with biotinylated DNA probe sets. (*A*) Plasmid pPW519-1R (6-kb insert) hybridized to a normal lymphocyte metaphase spread. Signals are located on the termini of 21q [see 4',6-diamidino-2-phenylindole (DAPI)-stained chromosomes in *Inset*] as verified by DAPI banding (not shown). (*B* and *C*) Normal human lymphocyte metaphase (*B*) and nuclei (*C*) after hybridization with the 94-kb plasmid pool probe set. The terminal band 21q22.3 is specifically labeled. (*D* and *E*) Signals on trisomy 21 (47,+21) lymphocyte metaphase spreads after hybridization with the 94-kb probe set (*D*) or chromosome 21 library DNA inserts with the CISS hybridization (14) protocol (*E*). Three chromosomes 21 are entirely delineated by the library inserts; additional minor signals (see text) are indicated by arrowheads (also in *G*). (*F–J*) Labeling of trisomy 21 lymphocyte nuclei by the library inserts (*F* and *G*; compare with *E*) and

21q22.3 and excluding Down syndrome as a possible diagnosis. A small increase of nuclei with three and four signals (both <5%) over that of normal lymphocytes was also observed, probably reflecting a higher portion of tetraploid cells in such CV samples.

The diagnostic potential of the chromosome 21 probes was further tested by using a glioma tumor cell line, TC620, known to be pseudotriploid with a highly rearranged genome (13, 15, 28). The metaphase spreads revealed two apparently normal chromosomes 21 and one translocation chromosome (see Fig. 1 *N* and *O*). Interestingly, the chromosome 21 DNA on the translocation chromosome labeled by the library probe has a size equivalent to a normal 21q region, thus suggesting a Robertsonian translocation event. However, fine structural aberrations of 21q (i.e., small deletions etc.) cannot be excluded by this analysis. The interphase signals seen with both the plasmid probe set and the library inserts were consistent with trisomy 21q22.3 and trisomy 21, respectively.

Finally, we determined if the 94-kb plasmid probe set could be used to localize chromosome 21 DNA sequences in solid tissues. Fig. 2 shows the nucleus of a cortical neuron from "normal" human brain tissue after *in situ* hybridization. Both chromosomes 21 are clearly labeled by the probe, and they are located near the nucleolus; this nuclear location is consistent with the fact that chromosome 21 contains a ribosomal gene cluster that is usually localized in the nucleolus. This observation suggests that these probes may also prove useful for evaluating the frequency of chromosome 21 mosaicism in specific cell or tissue types. In addition, it should be of interest to see if the various karyotypic changes associated with the Down syndrome phenotype alter the normal nuclear topography of chromosome 21 in neuronal tissue.

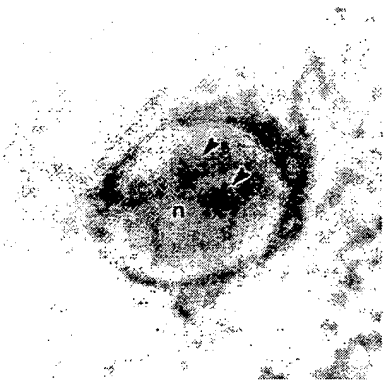

FIG. 2. Visualization of 21q22.3 in a nucleus of a large neuron in human parietal lobe tissue. The biotinylated plasmid pool probe set (94 kb) was detected by the purple-colored precipitate generated by alkaline phosphatase (conjugated to avidin). The arrows indicate the two signals; "n" indicates the center of the nucleolus.

DISCUSSION

We have demonstrated a method to rapidly detect numerical and structural aberrations of chromosome 21 in metaphase and in interphase cells. A trisomic karyotype can be diagnosed easily in interphase cells because the majority of the nuclei (55–65%) exhibit three distinct foci of hybridization. In contrast, less than 0.2% of nuclei in lymphocytes with a disomic karyotype show three nuclear signals; interestingly, the percentage of such nuclei in normal CV cells was higher but still considerably less than 5%. In general, as few as 20–30 cells were sufficient to unambiguously distinguish between disomic and trisomic cell populations. However, in view of the uncertainty of the level of chromosome 21 mosaicism in clinical samples, the number of cells required to make an unambiguous diagnosis will likely be higher. Additional clinical correlations will be required to establish the absolute number. Nevertheless, this analytical approach could allow the diagnosis of Down syndrome without the need to culture cells or to obtain metaphase spreads.

Pools of plasmid DNA from 21q22.3 and a "complete" set of DNA inserts from a chromosome 21 library were compared as probes. In general, the plasmid probe set was superior for interphase diagnosis because smaller and more focal areas were labeled with improved spatial resolution. This probe set, which labels 21q distal to the centromere, also has the particular advantage of relative insensitivity to interindividual pericentromeric heteromorphisms. Furthermore, unlike the library DNA inserts, there were no minor nonspecific hybridization signals. Eventually, removal of the cross-hybridizing sequences from the library DNA [e.g., as by subtractive hybridization (33)] could make chromosomal decoration from pter to qter more attractive for diagnostic purposes. It should also be noted that the 94-kb plasmid probe exhibited several types of hybridization patterns in interphase cells, each exhibiting subtle differences in structural detail (see Fig. 1 *C* and *H–K*). For example, in many G_1-phase cells, each nuclear domain can be resolved as a doublet of closely juxtaposed signals. The variability in the nuclear signal patterns may reflect dynamic changes in this segment of euchromatic DNA that occur at different stages of the cell cycle or during transcriptional activation.

Although we have used here selected plasmid clones containing only unique human DNA sequences, cosmid clones containing repetitive sequences can also be used to specifically label their cognate genomic region in metaphase and interphase cells by applying hybridization protocols like CISS hybridization that suppress the signal contribution of repetitive sequence elements (14, 34, 35). Therefore, single or nested sets of cosmids could be used as diagnostic tools for other genetic diseases in a fashion similar to that reported here. Trisomy of chromosomes 13, 18, 21, X, and Y together account for the vast majority of numerical and/or structural chromosome abnormalities identified during prenatal karyotyping. With the continued development of multiple nonisotopic probe labeling and detection systems (13, 15), it should be possible to visualize three or more chromosomes simultaneously following *in situ* hybridization. Thus, the development of a rapid and automated screening test to detect the major trisomic disorders directly in interphase cells from amniotic fluid or chorionic villi cells is a viable future objective. The analysis of specific human chromosomes by *in situ* hybridization has already been used to complement conventional cytogenetic studies of highly aneuploid tumor

the 94-kb probe set (*H–J*; compare with *D*). (*K–M*) Nuclei (*K*) and metaphase spread (*M*) of chorionic villi cells containing a translocation chromosome but showing a balanced karyotype with regard to 21q22.3, and DAPI-counterstained metaphase (*L*) indicating (lower arrow) the terminal region of 21q, which is translocated to a B-group chromosome independently identified as chromosome 4 by banding analysis (T. Yang-Feng, Yale University, personal communication). (*N* and *O*) Chromosome 21 delineated in a metaphase spread of the oligodendroglioma-derived cell line TC620 by using the library inserts probe (*O*) and DAPI staining (*N*). TC620 exhibits two chromosomes 21 and one translocation chromosome (see *N*, left arrow) whose short arm contains an apparently complete 21q, suggesting a Robertsonian translocation event. Arrows indicate either hybridization signals or the corresponding DAPI-stained chromosome. Detection of the hybridized probes was with fluorescein isothiocyanate-conjugated avidin [the signal of the 6-kb probe (*A*) was amplified (31)]. Similar results were obtained when the detection was mediated by alkaline phosphatase-conjugated avidin (not shown).

lines (15), and the extension to prenatal diagnostic applications seems warranted.

The analysis of karyotypes with translocations of chromosome 21 shows the usefulness of a regional probe set to rapidly identify and characterize even small translocations by unambiguous signals on metaphase chromosomes, thus circumventing an extensive analysis by high-resolution banding. In contrast, the library insert probe is more suitable for defining the relative amount of chromosome 21 DNA that has been translocated. By analyzing interphase nuclei, one can also determine if a balanced or unbalanced number of chromosomal regions exists. However, the detection of a translocated chromosome directly in nuclei would require double-labeling techniques to identify the recipient chromosome to which the chromosome 21 material was translocated. With prior knowledge of the chromosomes in question, such translocation events could be assessed by measuring the juxtaposition of the nuclear signals (9).

Finally, we have shown that probes containing 6 kb of sequence can be localized in both metaphase spreads and interphase cells with high efficiency. This detection sensitivity with nonisotopic reagents is similar to that achieved in other recent reports (36–38). The combination of nonisotopic *in situ* hybridization with DAPI or BrdUrd banding or total chromosome decoration with library DNA probes thus provides a simple and general approach for gene mapping. It also should facilitate the use of small DNA probes to rapidly pinpoint the breakpoints on translocation chromosomes, which could further aid in defining the genomic segment critical for Down syndrome.

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1. Lejeune, J. (1959) *Ann. Genet.* **1**, 41–49.
2. St. George-Hyslop, P. H., Tanzi, R. E., Polinsky, R. J., Haines, J. L., Nee, L., Watkins, P. C., Myers, R. H., Feldman, R. G., Pollen, D., Drachman, D., Growdon, J., Bruni, A., Foncin, J.-F., Salmon, D., Frommelt, P., Amaducci, L., Sorbi, S., Piacentini, S., Stewart, G. D., Hobbs, W. J., Conneally, P. M. & Gusella, J. F. (1987) *Science* **235**, 885–890.
3. Goldgaber, D., Lerman, M. I., McBride, O. W., Saffiotti, U. & Gajdusek, D. C. (1987) *Science* **235**, 877–880.
4. Tanzi, R. E., Gusella, J. F., Watkins, P. C., Bruns, G. A. P., St. George-Hyslop, P., Van Keuren, M. L., Patterson, D., Pagan, S., Kurnit, D. M. & Neve, R. L. (1987) *Science* **235**, 880–884.
5. Wisniewski, K. E., Wisniewski, H. M. & Wen, G. Y. (1985) *Ann. Neurol.* **17**, 278–282.
6. Selkoe, D. J. (1988) *Annu. Rev. Neurosci.* **12**, in press.
7. Watkins, P. C., Tanzi, R. E., Cheng, S. V. & Gusella, J. F. (1987) *J. Med. Genet.* **24**, 257–270.
8. Hamerton, J. L. (1981) in *Trisomy 21*, eds. de la Cruz, F. F. & Gerald, P. S. (University Park Press, Baltimore), pp. 99–107.
9. Rappold, G. A., Cremer, T., Hager, H. D., Davies, K. E., Müller, C. R. & Yang, T. (1984) *Hum. Genet.* **67**, 317–325.
10. Schardin, M., Cremer, T., Hager, H. D. & Lang, M. (1985) *Hum. Genet.* **71**, 281–287.
11. Manuelidis, L. (1985) *Hum. Genet.* **71**, 288–293.
12. Pinkel, D., Gray, J. W., Trask, B., van den Engh, G., Fuscoe, J. & van Dekken, H. (1986) *Cold Spring Harbor Symp. Quant. Biol.* **51**, 151–157.
13. Cremer, T., Tesin, D., Hopman, A. H. N. & Manuelidis, L. (1988) *Exp. Cell Res.* **176**, 199–220.
14. Lichter, P., Cremer, T., Borden, J., Manuelidis, L. & Ward, D. C. (1988) *Hum. Genet.*, in press.
15. Cremer, T., Lichter, P., Borden, J., Ward, D. C. & Manuelidis, L. (1988) *Hum. Genet.*, in press.
16. Cremer, T., Landegent, J., Brückner, A., Scholl, H. P., Schardin, M., Hager, H. D., Devilee, P., Pearson, P. & van der Ploeg, M. (1986) *Hum. Genet.* **74**, 346–352.
17. Julien, C., Bazin, A., Guyot, B., Forestier, F. & Daffos, F. (1986) *Lancet* **ii**, 863–864.
18. Moisan, J. P., Mattei, M.G., Baeteman-Volkel, M. A., Mattei, J. F., Brown, A. M. C., Garnier, J. M., Jeltsch, J. M., Masiakowsky, P., Roberts, M. & Mandel, J. L. (1985) *Cytogenet. Cell Genet.* **40**, 701–702 (abstr.).
19. Tanzi, R., Watkins, P., Gibbons, K., Faryniarz, A., Wallace, M., Hallewell, R., Conneally, P. M. & Gusella, J. (1985) *Cytogenet. Cell Genet.* **40**, 760 (abstr.).
20. Van Keuren, M. L., Watkins, P. C., Drabkin, H. A., Jabs, E. W., Gusella, J. F. & Patterson, D. (1986) *Am. J. Hum. Genet.* **38**, 793–804.
21. Nakai, H., Byers, M. G., Watkins, P. C., Watkins, P. A. & Shows, T. B. (1987) *Cytogenet. Cell Genet.* **46**, 667 (abstr.).
22. Münke, M., Foellmer, B., Watkins, P. C., Cowan, J. M., Carroll, A. J., Gusella, J. F. & Francke, U. (1988) *Am. J. Hum. Genet.* **42**, 542–549.
23. Masiakowski, P., Breathnach, R., Bloch, J., Gannon, F., Krust, A. & Chambon, P. (1982) *Nucleic Acids. Res.* **10**, 7895–7903.
24. Watkins, P. C., Tanzi, R. E., Gibbons, K. T., Tricoli, J. V., Landes, G., Eddy, R., Shows, T. B. & Gusella, J. F. (1985) *Nucleic Acids Res.* **13**, 6075–6088.
25. Watkins, P. C., Watkins, P. A., Hoffman, N. & Stanislovitis, P. (1985) *Cytogenet. Cell Genet.* **40**, 773–774 (abstr.).
26. Kaplan, J. C. & Carrit, B. (1987) *Cytogenet. Cell Genet.* **46**, 257–276.
27. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
28. Manuelidis, L. & Manuelidis, E. E. (1979) in *Progress in Neuropathology*, ed. Zimmerman, H. M. (Raven, New York), Vol. 4, pp. 235–266.
29. Manuelidis, L. & Borden, J. (1988) *Chromosoma* **96**, 397–410.
30. Brigati, D. J., Myerson, D., Leary, J. J., Spalholz, B., Travis, S. Z., Fong, C. K. Y., Hsiung, G. D. & Ward, D. C. (1983) *Virology* **126**, 32–50.
31. Pinkel, D., Straume, T. & Gray, J. W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2934–2938.
32. Devilee, P., Cremer, T., Slagboom, P., Bakker, E., Scholl, H. P., Hager, H. D., Stevenson, A. F. G., Cornelisse, C. J. & Pearson, P. L. (1986) *Cytogenet. Cell Genet.* **41**, 193–201.
33. Welcher, A. A., Torres, A. R. & Ward, D. C. (1986) *Nucleic Acids Res.* **14**, 10027–10044.
34. Sealy, P. G., Whittaker, P. A. & Southern, E. M. (1985) *Nucleic Acids Res.* **13**, 1905–1922.
35. Landegent, J. E., Jansen in de Wal, N., Dirks, R. W., Baas, F. & van der Ploeg, M. (1987) *Hum. Genet.* **77**, 366–370.
36. Lawrence, J. B., Villnave, C. A. & Singer, R. H. (1988) *Cell* **52**, 51–61.
37. Bhatt, B., Burns, J., Flannery, P. & McGee, J. O. (1988) *Nucleic Acids Res.* **16**, 3951–3961.
38. Albertson, D. G., Fishpool, R., Sherrington, P., Nacheva, E. & Milstein, C. (1988) *EMBO J.* **7**, 2801–2805.